# EXERCISE TRAINING REVERSES AGE-INDUCED INDUCIBLE NITRIC OXIDE SYNTHASE UPREGULATION

A Dissertation

by

WOOK SONG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2003

Major Subject: Kinesiology

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# ABSTRACT

Exercise Training Reverses Age-Induced

Inducible Nitric Oxide Synthase Upregulation. (December 2003)

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The risk of injury, inflammation, and oxidative stress increases in skeletal muscle with aging. It has been postulated that pro-oxidant signaling, including upregulation of inducible nitric oxide synthase (iNOS) contributes to inflammation, pathology, and aging in the brain, liver and heart. Exercise training reduces the risk of injury and inflammation. The purpose of this study was: 1) to identify the mechanisms that upregulate iNOS, pro-oxidant and pro-inflammatory signaling in skeletal muscle, and 2) to identify the mechanisms by which exercise training reduces pro-oxidant signaling. Protein levels and activity of iNOS were measured in 4 groups of male Fischer-344 rats (5 mo and 24 mo, n=10/group), old-control (OC), old-trained (OT), young-control (YC), and young-trained (YT). Exercise training protocol was 60 min at 15 m/min at 15° incline for 5 d/wk for 12 wk. Both iNOS protein expression and activity were significantly higher in OC compared to YC, but exercise training reversed the elevation of iNOS levels lower than OC in tibialis anterior. Surprisingly, NF-κB DNA binding activity was significantly lower in OC than YC, while increased with exercise training in white and red gastrocnemius in both OT and YT. In contrast, protein expression of p65, a regulatory subunit of NF-κB was significantly greater in OC than YC, while exercise training significantly reduced p65 in OT compared to OC from the white gastrocnemius. These data indicate that regulation of

NF-κB activity with aging is post-translational and alterations in iNOS expression may result from alternative NF-κB pathways. As decreased NF-κB activity with aging could result in downstream increase in pro-apoptotic signaling, we tested follow-up hypotheses that aging would increase pro-apoptotic regulator Bax and decrease the anti-apoptotic regulator Bcl-2. Bax increased while Bcl-2 decreased in OC in white gastrocnemius when compared to YC. In contrast, exercise training resulted in a dramatic upregulation of Bcl-2 and downregulation of Bax protein expression in OT when compared to OC. These novel results indicate that alterations in pro-inflammatory and pro-apoptotic signaling occur in skeletal muscle during the aging process. Importantly, our findings strongly support the hypothesis that exercise training reverses age-induced changes in pro-inflammatory and pro-apoptotic signaling.

# DEDICATION

To my loving wife, Youngshin, who has always accompanied me as a precious friend with love, understanding, and patience; to my wonderful daughter and son, Christy and Joshua, who mean joy and happiness to me; to Mom and Dad for their love, direction, encouragement, and all support; to my present and former advisors, who have given insights, challenges, and continuous encouragement; and finally to my Lord, Jesus Christ, who provides for every single need in my life.

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# CHAPTER I

# INTRODUCTION

Growing evidence suggests that production of nitric oxide (•NO) by nitric oxide synthase (NOS) is important in controlling skeletal muscle contractions, blood flow, glucose uptake, and metabolism (14). There are three isoforms of NOS in skeletal muscle. Neuronal NOS (nNOS) is located in the dystroglycan complex located at the cell membrane and also dispersed through the sarcolemma. Endothelial NOS (eNOS) is associated with mitochondria in skeletal muscle. Lastly, inducible nitric oxide synthase (iNOS) is normally expressed at very low levels in skeletal muscle (135), but can be dramatically upregulated through the transcription factor NF-κB and/or pro-inflammatory cytokines (59, 97). Low to moderate levels of nitric oxide are important in cellular homeostasis and prevent free radical-related damage. However, at high levels, chronic production of •NO represents a formidable vehicle for pathology. Reid (135) suggested a biphasic response for nitric oxide on $Ca^{2+}$ release dependent on nitric oxide concentration and time of exposure.

Recent studies indicated that overproduction of nitric oxide may be involved in muscle wasting, or cachexia, consistently observed with chronic heart failure, cancer, AIDS, and sepsis (2, 3, 29, 65). Elevated iNOS levels have also been detected in muscular dystrophy (22). Overproduction of nitric oxide may play a role in the etiology of muscle crush injury as well (141). Furthermore, nitric oxide production is greatly enhanced concomitant with muscle damage following 200 eccentric contractions in human skeletal muscle (133). Recently, Chung et al. (35) have found that iNOS is upregulated in kidney, heart, and brain with aging. However, the existence and role of iNOS upregulation in aging skeletal muscle is poorly understood.

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iNOS levels may be regulated by upstream pro-inflammatory, pro-oxidant signaling involving the activation of the transcription factor NF-κB. Transcription factors are proteins that control a number of genes and their ability to direct the manufacturing of new proteins and peptides. The inhibitory protein IkappaB (I-κB) normally binds with NF-κB, keeping it quiescent in the cytosol. Inflammatory cytokines (e.g., tumor necrosis factor-alpha: TNF-α, interleukin-1beta: IL-1ß), immune response to infection, injury, and oxidative stress can activate NF-κB via the phosphorylation and release of I-κB from NF-κB (59, 114). Once released, NF-κB translocates to the nucleus where it binds with DNA (i.e., activation) governing release of pro-inflammatory proteins and peptides (39). Thus, it is thought that NF-κB activation as an amplifier system for inflammation. NF-κB activation is indeed a potent amplifier of iNOS protein production (179).

Skeletal muscle function is known to decline with age, while the risk of modern chronic disease increases (26). Aging in skeletal muscle can be characterized as an increased susceptibility of skeletal muscle to inflammation and damage (177). One of the pathways important in the inflammatory process is “oxidative stress”. Oxidative stress occurs when oxidant production overwhelms the antioxidant system. A potential mechanism through which oxidant production can increase with inflammation and thus may contribute to skeletal muscle atrophy and weakness is iNOS, possibly activated through the transcription factor NF-κB. (2, 29).

## Review of literature

### 1. Aging in skeletal muscle

#### *1.1 Physical inactivity*

Physical inactivity is increasingly accepted as a significant contributor to age-related decline in physical function, increase in chronic disease risk, and premature mortality (25, 113). Declines in exercise capacity throughout an individual's life span can affect skeletal muscle function and can ultimately limit the ability to perform the activities of daily living. Indeed, some of the losses of strength, endurance, and glucose metabolism associated with aging are simply the product of inactivity (26, 63). Physical inactivity is now believed to be a critical contributor to modern chronic diseases including heart disease, high blood pressure, type II diabetes, chronic obstructive pulmonary disease, and cancer (26). Booth et al. (26) proposed that inactivity is a "pathology" in which alterations in the expression of critical genes and proteins occur that promotes the etiology of modern chronic diseases. Loss of skeletal muscle and lean body mass has been linked with increased risk of heart disease, hypertension, Type II diabetes, functional decline, and frailty (23, 49, 63, 112, 113, 130). Further, the presence of modern chronic disease exacerbates skeletal muscle dysfunction leading to a downward spiral of health (112).

#### *1.2 Susceptibility to inflammation*

Along with these changes in exercise capacity and function, a number of investigations reported age-associated changes in several parameters of immune status such as a decline in cell-mediated and humoral immunity (52). While resistance to infection from viruses and bacteria decrease with age, the prevalence for autoimmune disorders, chronic inflammatory disorders, and oxidative stress increase (172). In skeletal muscle, it has been demonstrated that selective

upregulation of skeletal muscle transcripts involved in inflammation and oxidative stress (84). Kavo et al. (84) reported that gene expression of NF-κB, cytokines and receptors, and other pro-inflammatory response markers were upregulated with aging in the rhesus monkey vastus lateralis. Consistently, cachexia or frank muscle wasting found in septic infections, a wide variety of diseases and aging is accompanied by an increase in cytokines, acute-phase proteins, and inflammation (93).

Skeletal muscles in old animals are far more susceptible to inflammation, injury, and muscle wasting (12, 116, 176, 177). A much greater loss of skeletal muscle mass occurred in old rats when compared to young adults after 4 weeks of immobilization and 4 weeks of subsequent recovery (176). Repeated eccentric contractions induce more tissue damage, force reduction, necrosis, and phagocytic invasion increase in skeletal muscle with increasing aging (116). Zerba et al. (177) demonstrated that old mice suffer greater muscle damage from eccentric contractions than do younger counterparts, and implicated an increased age-effect of reactive oxygen species (ROS). Higher plasma concentrations of pro-inflammatory cytokines IL-6 and TNF-α were associated with lower muscle mass and lower muscle strength in older men and women, indicating cytokines may contribute to the loss of muscle mass and strength that accompanies aging (167). Indeed, age-related increases in inflammation and susceptibility to muscle damage and wasting are proposed as linked to increased oxidative stress (116, 176). Similar effects of age on muscle damage have been also reported in humans (61). Baldwin et al. (12) recently reported that anti-inflammatory therapy attenuated muscle injury, strength loss, and soreness following eccentric exercise in older individuals.

*1.3 The inflammation hypothesis of aging*

It has been shown that there is an increased susceptibility of skeletal muscle to inflammation and tissue damage with aging (116, 177). While the immune system is often thought to decline with aging, inflammatory process, disease, and auto-immune disorders increase with age (172). For example, increased inflammation in the brain is now recognized as a critical factor in the aging process, Alzheimer's disease, and Parkinson's disease (117).

Based upon evidences accumulated and new data published recently, Byung Pal Yu and colleagues proposed the "Inflammatory Hypothesis of Aging," to highlight the involvement of the underlying inflammation process during the aging process (35). Even though the involvement of the inflammatory process in many diseases has long been known, its implication in the aging process has not been widely considered until a recent proposal, the "Inflammatory Hypothesis of Dementia," highlighting the importance of inflammation in aging (118). Mediators proposed to contribute to increased inflammation with aging and modern chronic diseases include pro-inflammatory cytokines such as TNF-α and IL-1β, NF-κB, cyclooxygenase-2 (COX-2), and iNOS (35, 117, 118). Specifically, Chung et al. (35) reported that age-related increases in the kidney, heart, and brain in the pro-inflammatory mediators including TNF-α, IL-1β, NF-κB, and iNOS that are reversed by caloric restriction, which also increased lifespan. This provides an underlying molecular insight into the anti-inflammatory action of caloric restriction in retarding the aging process.

*1.4 Oxidative stress in the aging process*

In 1956, Denham Harman first proposed "The Free Radical Theory of Aging" which postulates that the aging process is a complex result of the repeated toxic insults of free radical or oxidative stress. Today it is thought that oxidative stress increases in most tissues with aging

including skeletal muscle (78, 148). Increased production of oxidants with age could occur through (a) increased production of reactive oxygen species (ROS) by the electron transport chain, and (b) upregulation of iNOS (21, 117, 176). Indirect markers of oxidative stress such as lipid peroxidation are increased in skeletal muscle with aging (78, 104). In addition, Sohal et al. (149) measured 125% increase in mitochondrial $O_2^{\bullet -}$ production and a 21% increase in $H_2O_2$ production with aging. Bejma and Li (21) demonstrated that DCFH (dichlorofluorescein) oxidation, a direct marker of oxidant production, is higher in skeletal muscle from old rats when compared to young adults. In the heart, ROS production was also increased with age, but the acute exercise bout significantly increased ROS generation only in the old rat (20). These data clearly reveal that as aging progresses, a relatively small work task can provoke a greater ROS-generating effect in the heart and skeletal muscle.

A key contributor important in inflammation, especially with aging, that has received increasing attention is "oxidative stress" (116). Oxidative stress occurs when production of ROS and reactive nitrogen species (RNS) overwhelms the endogenous antioxidant system of enzymes (i.e., superoxide dismutase, glutathione peroxidase) and substrates (i.e., Vitamin E, reduced glutathione). EDL (extensor digitalis longus) muscle from aged mice was more susceptible to lengthening contraction than that from young mice, and treatment of superoxide dismutase alleviated muscle force deficit due to eccentric injury especially in the aged mice (177). It has been also documented that aged individuals are more susceptible to muscle injury (28), while inflammatory response is considered critical in the recovering from muscle injury, oxidants released from neutrophils can also cause secondary damage. Tidball (160) suggested that the dynamic balance between inflammatory cells and muscle-derived free radicals can play important roles in modulating the course of muscle injury and repair after modified loading.

ROS can be important in the initiation of exercise-induced muscle damage and in the initiation and propagation of the subsequent acute muscle inflammatory response (162). Oxygen radicals generated via the neutrophil respiratory burst are vital in clearing away muscle tissue that has been damaged by eccentric exercise and they may also be responsible for propagation of further damage (162). Finkel and Holbrook (54) have proposed that oxidative stress is secondary to recruitment of inflammatory cells which contain the potent NADPH oxidase system, which produces large amounts of superoxide. The NADPH oxidase complex is present in neutophils, macrophages, microglia and vascular cells and cell membranes. Consequently, in both the brain and the vessel wall, the production of superoxide reacts rapidly with nitric oxide leading to the formation of peroxynitrite. Overproduction of peroxynitrite is also known to be a causative factor of tissue-damage, particularly in the case of chronic inflammation (90). Due to the sustained effect of ROS and RNS, chronic inflammation is characterized by the infiltration of inflammatory cells such as lymphocyte and macrophage, and it is thought that ROS and RNS are playing as major role in inflammatory reaction.

A reduction in the responsiveness of cytoprotective proteins such as heat shock proteins (HSPs) can also contribute to enhanced susceptibility to damage and dysfunction from oxidative stress (116). Thus aging results in an imbalance between oxidant production and antioxidant protection. Moreover, aging mammals produce proportionally more protein which have errors in amino acid sequencing (140), and are consequently more susceptible to oxidation of amino acid residues and subsequent proteolysis (4) and free radicals have been implicated as contributing factors to age-dependent declines in proteolytic capacity (157). In order to study the molecular mechanisms associated with the decline in stress tolerance that accompanies aging, 207 stress-related genes were analyzed using a cDNA array showing that aging resulted in selective

upregulation of transcripts involved in cell growth, death, and signaling, along with a downregulation of genes involved in antioxidant defenses (178).

*1.5 Exercise training with age*

Exercise training is an efficient way of reducing the susceptibility of muscles to further exercise-induced damage and it has been suggested that this protection is associated with an increased activity of muscle antioxidant enzymes, including superoxide dismutase, catalase, and glutathione peroxidase (66, 69, 116, 139).

Physical exercise in adults, including the elderly, increases lean body mass (i.e., weight training in humans), decreases percentage body fat, and reduces the incidence of obesity, improves glucose metabolism, immune function, endurance and strength (50, 48, 88, 152). Booth et al. (26) proposed that exercise training can reduce the risk of most modern chronic diseases simultaneously. Chronic exercise training resulted in lower levels of lipid peroxidation in skeletal muscle (106) as well as aged myocardium (51). Muscle mitochondria showed improved mitochondrial respiratory control (34). These finding demonstrate that the adaptability of muscle to training in the young animals does not vanish at old age. Endurance training can also effectively restore age-related deterioration of muscle protein content and mitochondrial oxidative capacity (55). However, heavy training may cause a deficit in muscle antioxidant reserve and protective margin (153, 60).

It appears that ROS/RNS production increase significantly while antioxidant enzymes are also elevated (79, 44) with little change in HSPs during aging process in skeletal muscle. Therefore, upregulation of antioxidant and stress protein defenses with age seem to be insufficient, as a result, oxidative stress occurs with aging.

While many of the gross physical properties of skeletal muscle that occur with aging and exercise training are fairly well understood, little is known about the mechanistic cell-signaling pathways that contribute to loss of skeletal muscle function with age and improvement with exercise training.

## 2. Inducible NOS (iNOS) in skeletal muscle

### *2.1 NOS isoforms in skeletal muscle*

The presence of both •NO production and identification of NOS isoforms in skeletal muscle were first described by Balon and Nadler (15) and Kobzik et al. (89), respectively. The initial NOS isoforms which have been identified in skeletal muscle are nNOS (neuronal) and eNOS (endothelial), both of which are $Ca^{2+}$-dependent (135). The nNOS isoform is expressed at higher levels in fast twitch muscle fibers when compared to slow-twitch fibers (135), and is found near the sarcolemma where it is integrated into the cytoskeletal matrix and bound to such proteins as dystrophin and integrins and these are part of the focal adhesion complex (62). eNOS has been associated with mitochondria in rodent models (89) co-localizing with mitochondrial markers, and is abundant in skeletal muscle vasculature (56).

iNOS is an another isoform that is normally expressed at very low levels in skeletal muscle (135), but can be dramatically increased by pro-inflammatory cytokines such as TNF-α and IL-1β (97). From low to moderate levels of nitric oxide has been shown to protect against oxidative stress related damage, but at high levels, increased NO by iNOS could contribute to formidable pathology suggesting a biphasic response of nitric oxide on $Ca^{2+}$ release dependent on concentration and exposure time (135). iNOS levels may be elevated during muscle inflammation directly by leukocytes or by activation of NF-κB. Cytokines may increase iNOS (i) directly through the cytokine response element, or (ii) through an upregulation of NF-κB, or (iii)

by phosphorylation and degradation of I-κB, lifting suppression of NF-κB (29, 59). In addition, nitric oxide production by inducible isoform has been implicated in muscle dysfunction (77, 94, 159).

*2.2 Induction by LPS (lipopolysaccaride) and pro-inflammatory cytokines*

Upregulation of iNOS in response to pro-inflammatory cytokines has been investigated in cultured skeletal muscle cells (154). Combination of IL-1β and IFN-γ induced a marked accumulation of nitrite, a stable metabolite of nitric oxide, and iNOS mRNA (125) and protein expression was also increased in L6 rat skeletal muscle cells (18, 125). However, Williams et al. (173) demonstrated that neither LPS, IL-1β, TNF-α, nor IFN-γ was able to stimulate nitrite production by mouse C2C12 skeletal muscle cells when administered alone. In contrast, combinations of IFN-γ with either TNF-α or IL-1β resulted in increased nitrite production and yielded an mRNA band homologous to mouse macrophages iNOS suggesting that combinations of cytokines stimulate NO production in skeletal muscle cells via induction of iNOS gene. Recently, the combination of LPS and IFN-γ, but neither alone, increased human iNOS promoter activity 28-fold, and full activation of the human iNOS promoter by cytokine mixture (i.e., IFN-γ, IL-1β, and TNF-α) was also observed (96). It is important to note that many of pro-inflammatory proteins including iNOS, IL-1, IL-6, and TNF-α contain redox-sensitive NF-κB specific, DNA binding sites in the promoter regions and that their production is greatly influenced by the oxidative status (81).

It is also well known that iNOS is readily induced by LPS in skeletal muscle. Boczkowske et al. (24) reported that iNOS was induced in the diaphragm after LPS injection in rats, being involved in the decreased muscular force. In contrast, Comtois et al. (38) showed that treatment of LPS with iNOS knockout mice upregulated the expression of the neuronal NOS

eliciting a significantly greater decline force generated in response to high frequency of stimulation suggesting that iNOS may play a protective role in attenuating the inhibitory influence of LPS on muscle contractility.

*2.3 iNOS expression in normal, inflammatory conditions, and diseases*

Nitric oxide produced by iNOS plays a role in immune responses to tumors and bacteria (98), and the gene of iNOS is induced by endotoxin and cytokines. However, in normal skeletal muscle, very low levels of iNOS are expressed as iNOS cDNA sequences was detected by Northern blot or RT-PCR analyses (128). iNOS mRNA is undetectable or present at very low levels in rat skeletal muscle (77, 121). Similarly, recent published review paper summarized that immunoblot and immunocytochemical analyses showed no or only minimally detectable iNOS immunoreactivity in various skeletal muscles of rat and mouse (151), but it is important to note that most of studies above have been done in 90s and thereafter antibodies are so well developed that chances of iNOS detection in normal muscles have been significantly increased.

Nitric oxide compromises muscle function during generalized inflammation with the primary source of •NO from iNOS expressed by macrophages and/or muscle fibers (82). Increased •NO production during sepsis by endotoxin administration resulted in reduced force-generating capacity in skeletal muscle fibers (155). Maximal diaphragm mitochondrial oxygen consumption was also reduced in the same model (31). Consistent with the above findings, NOS inhibitors have been shown to reduce muscle inflammation and necrosis (129).

iNOS upregulation is a commonality among numerous pathologies and models that result is wasting of skeletal muscle. For example, iNOS expression was greatly increased in skeletal muscle of chronic heart failure patient (138). Adams et al. (3) reported that iNOS expression was increased five- to nine fold in skeletal muscle of patients with chronic heart

failure suggesting that iNOS may be linked with exercise intolerance seen in heart patients. In a follow-up study, Hambrecht et al. (65) reported that increased expression of iNOS in skeletal muscle of patients with chronic heart failure was inversely correlated with mitochondrial creatine kinase expression and exercise capacity. Adams et al. (2) reported that apoptosis is frequently found in skeletal muscle obtained from chronic heart failure patients and iNOS and Bcl-2 are possibly involved in the regulation of apoptosis.

It is of interest that growing evidence suggests that overproduction of nitric oxide may be involved in cachexia from a number of additional pathologies including cancer, AIDS, and sepsis (29). Buck and Chojkier (29) showed that the decreased body weight, muscle wasting and skeletal muscle molecular abnormalities of cachexia were prevented by treatment of TNF-α mice with the antioxidants D-α-tocopherol, or the NOS inhibitor nitro-L-arginine. Elevated iNOS levels have also been detected in muscular dystrophy (22). Muscle crush injury has been shown to be associated with activation of the •NO system mainly due to enhancement of iNOS suggesting that delayed induction of iNOS may cause devastating damage to muscle and contribute to rhabdomyolysis (141).

Inflammatory myopathies are also characterized by an upregulation of iNOS. In 21 patients with autoimmune inflammatory myopathies, muscle fibers in myositis displayed distinct increase of iNOS and suggested that enhanced expression of iNOS with production of nitric oxide may contribute to oxidative stress mediating muscle fiber damage and muscle fiber necrosis (158). The accumulation of nNOS and iNOS in abnormal muscle fibers and implication of oxidative stress have also shown in the pathogenesis of inclusion body myositis (7). Excessive production of •NO may contribute to the destruction of tissues in such chronic inflammatory processes in diabetes, arthritis, transplant rejection, and inflammatory bowel syndrome (37, 119).

*2.4 Effects of age on iNOS expression*

An imbalance between iNOS and other isoforms is proposed to occur with age (117, 176). Thus a rise in iNOS with a reduction in nNOS would indicate a shift from a contractile role to an inflammatory role for nitric oxide with aging (117). The ability to induce iNOS, and thereby generate large amounts of NO suggesting that iNOS may play a major role, as one might expect in a chronic condition such as aging (35). However, chronic upregulation of iNOS with during inflammation to fight off microorganisms and to prevent apoptosis needs to be differentiated with aging condition.

There has been mounting evidence that a shift of the balance of the immune system occurs with aging towards chronic production of pro-inflammatory cytokines. Recently, it has been shown that in older rats, an age-related increased sensitivity to inflammation with advanced age, and age-induced increase in mRNA levels of IL-1β and IL-6 was further enhanced by the LPS treatment indicating an increased sensitivity to inflammation with age (100). Reports showed that during aging a shift increases in the ration of native to memory T cells with associated changes in the cytokine profile toward the preferential production of pro-inflammatory IL-1β, IL-6, INF-γ, TNF-α, and TGF-β (124, 126, 169).

Poynter and Daynes (132) showed that mean daily urinary excretion of urinary nitrate plus nitrite was greater in aged mice than in young mice, and reflective of increased iNOS level. In addition, they found that splenocytes from aged mice produced high levels of iNOS mRNA in response to stimulation with LPS and IFN-γ suggesting that aged mice produce greater quantities of NO through the enhanced transcriptional upregulation of the iNOS gene. Cernadas et al. (33) have reported that blood vessels from aging rats showed a marked ability to produce •NO through the presence of the iNOS isoform compared to young rats and in the aging aortas, and the expression of iNOS was also enhanced. Recently, Chung et al. (36) proposed that the

increased iNOS gene expression during the aging process could causally be related to the chronically activated pro-inflammatory state. However, little is understood regarding iNOS expression with aging skeletal muscle.

Kaminski and Andrade (82) proposed that •NO may protect skeletal muscles from the deleterious effects of ROS generated over the life span, and consequently, the reduction of NOS activity (nNOS) in old skeletal muscle may make the muscle more susceptible to mechanical or oxidative damage. Contrary to this proposal, there are also evidence that thiol oxidation and accumulation of 3-nitrotyrosine, an oxidation product of RNS, of sarcoplasmic reticulum Ca-ATPase are increased by aging, indicative of "nitrosative damage" (143, 166). Apparently, loss of balance between ROS and •NO and cellular antioxidant systems can be critical to determine the onset of age-related oxidative and nitosative stress (82).

*2.5 Effects of acute exercise vs. chronic exercise training on iNOS expression*

While acute exercise appears to mediate pro-inflammatory effects, chronic endurance exercise training has been shown to act in an immunosuppressive way. Niess et al.(122) reported an increased expression of iNOS at the transcriptional and translational level in human leukocytes in response to vigorous acute running exercise (half-marathon lasting 90 min), reflecting an inflammatory response and may contribute to an exercise-induced rise of endogenous nitric oxide production. Similarly, acute exercise consisting of an incremental treadmill test followed by a continuous run until exhaustion at 110% of the individual anaerobic threshold increased iNOS protein in monocytes and granulocytes (123).

In contrast to acute exercise, Bagby et al. (11) demonstrated that TNF-α release is attenuated in response to bacterial LPS in rats with prior long-term exercise training. In a recent study, Hambrecht et al. (64) showed that chronic exercise training reversed the overexpression of

iNOS and enhances oxidative capacity of skeletal muscle in patients with chronic heart failure. They were also able to show that local expression of TNF-α and IL-1β that are potent activators of iNOS expression is reduced. Taken together, these results show that prolonged exercise training elicits a selective downregulation of the pro-inflammatory cytokine production in healthy subjects and patients with chronic heart failure, and confirm the anti-inflammatory effects of chronic exercise trainings. Schulze et al. (144) proposed mechanism that a reduction of local cytokine expression was associated with a reduced iNOS expression which may in turn contribute to disinhibition of aerobic enzymes by reduction of intracellular •NO accumulation and protein nitrosylation.

Effects of aging and exercise training on macrophage iNOS gene expression have been determined. IFN-γ and LPS treatment significantly increased iNOS gene expression in the young, but not the old mice suggesting that aging reduces, and exercise training enhances, the capacity of resident peritoneal macrophages to respond to IFN-γ and LPS and kill tumor cells (111). Similarly, Woods et al. (174) showed that exercise training can increase macrophage anti-tumor activity in mice of different ages. However, not all functions are enhanced by exercise, and exercise-induced reductions in macrophage MHC II expression and antigen-presentation capacity have been documented.

While iNOS expression is relatively well documented in pathologic conditions and *in vitro* studies, it is unknown about iNOS alterations during physiological aging process (*in vivo*) and effect of chronic exercise training on iNOS-induced pro-oxidant and pro-inflammatory signaling in aging skeletal muscle.

**3. NF-κB in skeletal muscle**

*3.1 A potential upstream mechanism of iNOS*

The NF-κB is a heterodimer (p105) composed of two subunits (p65 and p50). In non-stimulated cells, NF-κB resides in the cytoplasm as an inactive complex bound to its inhibitor, I-κB. Upon stimulation with various agents including cytokines, mitogenes, viruses and reactive oxygen intermediates, I-κB dissociates from the NF-κB/I-κB complex and translocates to the nucleus, binding with high affinity to specific sites in the promoter regions of target genes and stimulating their transcription (165).

Treatment of cells with various inducers results in the phosphorylaton, ubiquitination and subsequent degradation of I-κB proteins. Proteolytic cleavage of p105 results in two antagonist proteins: p50, which has DNA-binding activity but no transactivation domain, and the inhibitory I-κBγ protein. This results in the release of NF-κB dimers which subsequently translocate to the nucleus, where they activate appropriate target genes including iNOS. NF-κB can be activated by a number of stimuli, including components of bacterial cell walls, such as LPS, or inflammatory cytokines, such as TNF-α or IL-1β that may increase iNOS directly, or through an upregulation of NF-κB. Therefore, NF-κB activation can be regarded as a potential upstream mechanism of iNOS expression. Upstream activators of I-κB phosphorylation and NF-κB activation include IKK (I-κB kinase) phosphorylation by NIK (NF-κB inducing kinase), cytokine driven receptor dependent and independent signaling, ROS, and MEKK2 (mitogen activated protein kinase kinase) (39, 57, 59).

*3.2 Important transcription factor in chronic inflammation*

NF-κB increases the expression of the genes for many cytokines, enzymes, and adhesion molecules in chronic inflammatory diseases (9, 13, 17, 115). Specifically, NF-κB regulates transcription of pro-inflammatory gene such as TNF-α, IL-1β, adhesion molecules, and COX-2. Consequently, persistent NF-κB activation found in many chronic inflammatory diseases, including rheumatoid arthritis, atherosclerosis, asthma, and inflammatory lung and bowel diseases, plays a pivotal role in this process (114, 134). Indeed, Brand et al. (27) discovered the presence of activated NF-κB in human atherosclerotic tissue for the first time. Induction of chronic inflammation simulating rheumatoid arthritis produced strong NF-κB activity in the affected joints (32). This was revealed by in vivo imaging, which has been developed a versatile model for monitoring NF-κB activation in vivo.

NF-κB regulates the expression of several genes encoding adhesion molecules that recruit inflammatory cells from the circulation to the site of inflammation in chronic inflammatory diseases (17). In terms of regulatory role of NF-κB in mucosal inflammation, Jobin and Sartor (80) recently proposed that the stimulatory environment partially determines whether the effect of NF-κB is protective or deleterious for the host, and κB-dependent pro-inflammatory gene expression and adhesion molecules may be extremely important in early protective responses but, when dysregulated, could lead to the development of chronic inflammation.

The broad involvement of NF-κB in various aspects of the pathology of chronic inflammation makes it an extremely attractive target for therapeutic intervention. Various anti-inflammatory drugs, including antioxidants, glucocorticoids, immunosuppressants and plant compounds, act as inhibitors of the NF-κB pathway, suggesting that the suppression of NF-κB is

an essential part of their anti-inflammatory activity (47, 114). For example, it was reported that prevention of the activation of NF-κB by PDTC (proline dithiocarbamate), potent inhibitors of NF-κB, reduces the development of chronic inflammation in arthritis model (40).

*3.3 Inducement of iNOS expression by NF-κB activation*

Toga et al. (163) have found that iNOS was expressed and NF-κB was activated in alveolar type II cells playing a crucial role in the progression of lung inflammation and injury. It has been reported that a profound activation of NF-κB after endotoxic shock correlated with induction of iNOS (137). Baek et al. (8) have also shown that the mammalian group IIA secretory phospholipased A2, that is believed to play an important role in inflammation, induces iNOS in macrophages and this induction occurs through NF-κB activation. In the same context, recent studies have demonstrated that several anti-inflammatory phytochemicals inhibit iNOS expression by blocking improper NF-κB activation, and one of the plausible mechanisms underlying inhibition of NF-κB activation by repression of degradation of the inhibitory unit I-κBα, which hampers subsequent nuclear translocation of the functionally active subunit of NF-κB (156). Another study indicated that curcumin (i.e., an NF-κB inhibitor) strongly reduced mRNA levels of iNOS in LPS-activated macrophages and blocked the LPS-induced binding of NF-κB suggesting that curcumin may exert its anti-inflammatory property by suppressing the activation of NF-κB through inhibition of upstream of I-κB (127).

Very recently, Adams et al. (1) have conduced in vitro and in vivo study to determine mechanisms of the regulation of iNOS induction in skeletal muscle of patients with chronic heart failure, and the intracellular signal transduction leading to iNOS expression. They concluded that

in skeletal muscle the activation of the transcription factor NF-κB is essential for the induction of iNOS expression.

*3.4 ROS mediated NF-κB activation*

Recently, several studies with muscle cell lines confirmed that NF-κB activation is mediated by oxidative stress. Zhou et al. (180) demonstrated that oxidative stress led to increased DNA binding of NF-κB in differentiated muscle cells. Moreover, NF-κB is actively involved in the upregulation of gene expression in the glutathione peroxidase and catalase in response to oxidative stress. NF-κB activation mediated by ROS generation may provide a mechanism by which intracellular lipid accumulation occurs in skeletal muscle cells (30). Again, ROS stimulated IL-6 production from skeletal myotubes in a manner that involves transcriptional activation of the IL-6 gene through an NF-κB-dependent pathway (92).

Overall, it is well accepted that one of the unique characteristics of NF-κB is its exquisite sensitivity to oxidative stress. However, it has been recently suggested that there are other two key pathways to NF-κB activation, involving TNF-α and IL-1β, and neither pathway seems to have any requirement for ROS (10). It is also possible that NF-κB DNA binding activity may be increased through a signaling pathway that does not involve IKK or NIK (NF-κB inducing kinase). For example, inflammatory MAPK (mitogen activated protein kinase) pathways such as JNK (c-jun terminal kinase) and p38 pathways, induced by MEKK1 (mitogen activated protein kinase kinase), could contribute to ROS-related activation of NF-κB without help of IKK or NIK (39, 57, 59). In addition, NAD(P)H oxidase and xanthine oxidase, as ROS sources, could activate NF-κB directly. The NAD(P)H subunit $gp91^{phox}$ could be used as a

marker of NAD(P)H activity. The importance of NAD(P)H oxidase in activation of NF-κB could be teated by inhibition of NAD(P)H oxidase or $gp91^{phox}$ knockout.

The capacity of NF-κB as a transcription factor depends on interactions with different transcriptional co-activators (147). Although regulated independently, the pathways controlling I-κB degradation and NF-κB function act in synergy in the activation of NF-κB-mediated transcription (114).

*3.5 NF-κB and muscle atrophy (protein loss)*

It has been said that TNF-α is implicated in muscle atrophy associated with a variety of chronic diseases. Li et al. (108, 109) reported that TNF-α directly induces muscle protein degradation in differentiated skeletal muscle myotubes, where it rapidly activates NF-κB, and demonstrated that protein loss induced by TNF-α is NF-κB dependent suggesting that a key role of ROS in mediating NF-κB activation in muscle atrophy. In a follow-up experiment, Li and Reid (107) used mutant I-κBα proteins that selectively inhibit NF-κB activation and found that muscle proteins were unaltered by TNF-α in the dominant negative cell lines in contrast differentiated myotubes transfected with the empty vector underwent a drop in total protein content indicating that NF-κB is an essential mediator of TNF-α-induced catabolism in differentiated muscle cells. On the other hand, Langen et al. (102) reported that NAC (N-acetyl-l-cysteine) decreased TNF-α-induced activation of NF-κB only marginally, indicating that the redox-sensitive component of the inhibition of myogenic differentiation by TNF-α occurred independently, or downstream of NF-κB.

Recently, Zarzhevsky et al. (176) proposed a model for muscle wasting in limb immobilization showing that NF-κB is activated by oxidative stress to induce iNOS and

increased •NO possibly causes muscle proteolysis systems, i.e., ubiquitin-proteosome system, lysosomal degradation, and $Ca^{2+}$ dependent proteolysis. Hunter et al. (76) reported that seven days of hindlimb unloading led to a 10-fold activation of an NF-κB in rat soleus muscle. Whereas several NF-κB family members were upregulated, the prototypical markers of cytokine-induced activation of NF-κB seen with disease-related wasting were not evident during disuse atrophy. There has been some suggestion that genes encoding components of the ubiquitin-proteasome system are targets because many of these are upregulated in both cachexia and disuse atrophy (83).

*3.6 Effects of aging on NF-κB activation*

Aging causes an increase in constitutive nuclear binding activity of NF-κB in brain, as it does in heart, liver, and kidney (67). Helenius et al. (67) suggested that aging process might be regulated differentially in tissues and cultured fibroblasts, perhaps reflecting differences between mitotic and post-mitotic cells. In tissues, aging seems to involve specific changes in the regulation of NF-κB components and also changes in the DNA-binding affinities of the NF-κB complex. Enhanced oxidative stress during aging is accompanied by compensatory induction of the antioxidant enzyme HO-1 (HO: heme oxygenase) through activation of the NF-κB pathway in the liver (103). A similar finding was also reported in the kidney where age-related oxidative stress may be a primary cause of upregulated and altered NF-κB activity (85). Furthermore, the antioxidant action of caloric restriction normalized homeostasis of NF-κB/I-κB signaling pathway with aging.

Investigations have been done to elucidate the mechanisms by which NF-κB is activated in aging process. Accompanied with the change in the NF-κB activity was a decreased IκBα as confirmed by the increased nuclear p65 protein indicating that the aging process increases NF-

κB activity by downregulating IκBα (86). Kim et al. (85) proposed that NF-κB activation induces I-κB phosphorylation and degradation by the activation of I-κB kinase, which in turn trigger the activation NF-κB. Three pro-inflammatory enzymes in liver, COX-2, iNOS, and HO-1, showed an age-related increase, consistent with the NF-κB activation suggesting that under oxidative stress during aging, the inflammatory status of the aged organism would be more sensitized because of the enhanced responses by NF-κB (101). Moreover, in this study, the increased dissociation of I-κBα that occurred in cytosol facilitates the increased NF-κB translocation.

Alternative NF-κB activation pathways have been recently reported including cytokine response element and selective alterations in NF-κB subunits. For example, in the study showing that the activity of DNA binding complexes was significantly reduced in old animals, interestingly, the age-related decline in the activation of NF-κB could not attributed to an alteration in the composition of constituent subunits or degradation of NF-κB inhibitory proteins but rather was due to selective down-regulation of RelA/p65 and NF-κB2/p52 proteins (120). Additionally, although a significant age-related increase in NF-κB binding activity was founded in rat liver, aging did not affect the protein levels of the main I-κB subunits (68). It is possible that the mechanisms by which NF-κB activity is increased can be differentially regulated in tissue-specific manner or can be regulated by redundant pathways.

Only one result has been published that NF-κB activity was decreased with age in three muscle types, including gastrocnemius, vastus lateralis and soleus (71). In this study, however, i) protein levels of NF-κB was not reported, and ii) differential expressions of NF-κB subunits were not investigated.

Overall, the extent to which changes in NF-κB activity contributes to the aging process and life span in humans and other mammals is not clear. However, current thought is that changes in NF-κB activity observed with age are more safely categorized as an "effect" of aging, rather than a "cause." (58).

*3.7 Effects of acute exercise on NF-κB activation*

Current understanding of NF-κB activation is derived mostly from *in vitro* studies and very limited data is available regarding NF-κB activation with exercise. Growing evidences show that glutathione (GSH) plays a critical role in the maintenance of tissue antioxidant defenses and in the regulation of redox sensitive signal transduction. In muscle cells, the level and redox status of GSH regulates activity of the redox sensitive transcription factor NF-κB, and physical exercise may cause oxidation of GSH in tissues such as skeletal muscle, blood and liver (145).

Vider et al. (165) provided the first evidence to demonstrate that physical exercise (1h, 80% $VO_{2max}$) may trigger NF-κB activation in peripheral blood lymphocytes of physically fit young men. In this study, enhanced expression of TNF-α and IL-2 receptor was observed in plasma and elevated levels of lipid peroxidation was also reported in association with exercise-induced NF-κB activation. Another human study (171) reported an increased NF-κB binding activity after performing one hour maximal run on a treadmill.

NF-κB binding site is present in the promoter of the Mn-SOD (Manganese superoxide dismutase) gene, and oxidative stress has been shown to activate their binding (70, 170). With this context, the effect of acute exercise on SOD gene expression and the potential upstream mechanism of NF-κB were studied in rat skeletal muscle. Hollander et al. (72) found that NF-κB DNA binding activity significantly increased following a single bout of treadmill exercise in

both type IIA and type IIB muscles, which enhanced Mn-SOD mRNA transcription in deep vastus lateralis muscle.

Again, very limited data are available *in vivo* studies investigating the effect of acute exercise on NF-κB activation. Moreover, it is unknown about the effect of chronic exercise training on NF-κB activation in aging tissue, including skeletal muscle.

# CHAPTER II

# EXERCISE TRAINING REVERSES AGE-INDUCED INDUCIBLE NITRIC OXIDE SYNTHASE UPREGULATION

## 1. Introduction

Physical inactivity is now believed to be a critical contributor to "modern chronic diseases" including heart disease, Type II diabetes, hypertension, and cancer (26). Loss of skeletal muscle and lean body mass has been linked with increased risk of modern chronic diseases (49, 63, 113). There is growing evidence that chronic inflammation plays a major role in the pathology of such chronic diseases (5, 75). Similar to inactivity, aging is also characterized by an increase in susceptibility to injury and inflammation (177). It has been postulated that a critical mediator of inflammation with aging is iNOS (117). iNOS expression may be elevated during muscle inflammation by inflammatory cytokines (i.e., TNF-α and IL-1ß) and activation by NF-κB or directly through the cytokine response element. iNOS is normally expressed at extremely low levels in skeletal muscle (135), but can be dramatically upregulated by inflammatory cytokines (97). Cytokines also increase iNOS via phosphorylation of I-κB kappaB (Inhibitory kappa B), lifting its suppression of NF-κB (29, 59). A rise in iNOS with a reduction in nNOS (neuronal NOS) would indicate a shift from a contractile role to an inflammatory role for nitric oxide with aging (117). Unfortunately, little is understood about age-related changes in iNOS protein expression in skeletal muscle. Moreover, it is important to know whether aging upregulates NF-κB as a potential mechanism to increase iNOS levels. It has been shown that aging causes an increase in constitutive nuclear binding activity of NF-κB in brain, as it does in heart and liver (67). Exercise training in the absence of overtraining can reduce the risk of injury

and inflammation from mechanical and oxidant perturbations (52). However, there is virtually no information regarding exercise training and pro-inflammatory pathways in skeletal muscle, thus the influence of exercise training on iNOS expression and NF-κB activation in skeletal muscle remains unknown.

Therefore, the objectives of this study were to (i) determine the effects of aging and exercise training on iNOS protein expression and activity in rat skeletal muscle, and (ii) determine the upstream mechanisms by which iNOS levels are altered by measuring both NF-κB DNA-binding activity and I-κB expression. We hypothesized that iNOS protein expression and activity would increase with age, and exercise training would attenuate the elevation of iNOS expression. We further postulate that binding of NF-κB to DNA, a mechanism for elevated iNOS levels, would also increase with aging, but would decrease with exercise training. If aging increases iNOS and NF-κB binding activity in skeletal muscle, then these findings would provide further support for the global hypothesis that aging increases the inflammatory state, and thus increases the risk for weakness and muscle atrophy.

## 2. Methods

### *2.1 Animals*

We used 4-mo (young), and 25-mo (old) male Fischer-344 rats. Fischer-344 rats are the preferred NIH aging models with an average life span of 702 d. Animals have been purchased from the NIH colony and cared for at the LARR (The Laboratory Animal Resources and Research) facility at Texas A&M University in accordance with NIH and ULACC (The University Laboratory Care Committee) standards. Rats were housed in a temperature-controlled (23 ± 2°C) room with a 12:12-h light-dark cycle. Water and rat chow were provided *ad libitum*. The methods employed in this study were approved by ULACC in Texas A&M University.

### *2.2 Experimental design*

Experiment I: Our working hypothesis was that aging would increase iNOS levels, I-κB phosphorylation (degradation), NF-κB DNA binding activity and protein level of NF-κB subunit p65. To test the effect of aging and exercise training on iNOS expression of rat skeletal muscle, both iNOS protein expression and iNOS activity were measured in 4 groups (n=10/group), old-control (OC), old-exercise trained (OT), young-control (YC), and young-exercise trained (YT). iNOS and total NOS activity were determined by measuring the conversion of [$^{14}$C]arginine to [$^{14}$C]citrulline. iNOS and p65 protein levels were determined by Western immunoblot analysis.

Experiment II: Our working hypothesis was that exercise training would reverse age-induced changes in iNOS signaling by inhibition of IκB phosphorylation and degradation as upstream regulators. As a result, exercise training would ameliorate the elevation of NF-κB DNA binding activity in aging skeletal muscle. To determine the upstream mechanisms by which iNOS expression is altered, NF-κB DNA binding activity, I-κB protein expression, phosphorylation of I-κB were measured in 4 groups (n=10/group), OC, OT, YC, and YT.

Furthermore, to test the possible anti-apoptotic role of NF-κB, Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) protein expression were determined. NF-κB DNA binding activity was measured by new, sensitive ELISA technique that is specific for activated NF-κB. I-κB proteins expression and I-κB phosphorylation were measured by Western immunoblot analysis and phosphor-specific antibody was used to detect I-κB phosphorylation.

*2.3 Exercise training*

Treadmill running was the mode of exercise training. Rats in the exercise groups ran on a motor-driven treadmill at 15 m/min up a 15° incline, 1 h/d, 5 d/wk for 12 wk. This is low to moderate intensity exercise, the equivalent in human terms of a slow jog for an hour. The first 5 d were an acclimation period for rats to adapt to the treadmill machine without incline at 15 m/min for 10 min. Rats were gradually conditioned to perform the exercise at 15 m/min on 15° incline up to an hour over the first 5 wk of the 12 wk training program. This exercise regimen has previously been shown to elevate citrate synthase activity a marker of mitochondrial content (42, 43, 150). Heart weight / body weight ratio was assessed as an indicator of training state to determine the efficacy of the exercise training regimen.

*2.4 Muscle preparation*

Animals were anesthetized with sodium pentobarbital (120 mg/kg) 48 hours after the last day of training. Upon reaching a surgical plane of anesthesia, the soleus, gastrocnemius (red and white) and tibialis anterior muscles were quickly excised and immediately place in chilled (4°C) complete lysis buffer. The animals were then exsanguinated and disposed of in the LARR necropsy facilities. The lysis buffer (pH adjusted to 7.5) contains the following: 20 mM HEPES, 350 mM NaCl, 20% glycerol, 1% Igepal-CA630, 1 mM $MgCl_2$, 0.5 mM EDTA and 0.1 mM EGTA. DTT (10 mM) and protease inhibitor cocktail (Roche, Germany) are added fresh into

lysis buffer to make complete lysis buffer and used immediately. Once cleaned of excess connective tissue and fat, the muscles were quickly frozen in liquid nitrogen and stored at –80°C until analysis.

*2.5 Homogenization procedure*

Muscles were further minced into fine pieces, weighed and homogenized (20:1 w/v) in complete lysis buffer, maintaining temperature at 4°C throughout all procedures. Samples were homogenized in a Dounce glass homogenizer (Bellco Biotechnology: Vineland, NJ) and then centrifuged at 1,500 g to remove particulates. Three ml of complete lysis buffer used per 1 g of muscle tissue during homogenization. Protein concentration was measured at using BCA protein assay reagent kit (Pierce: Rockford, IL) at 562 nm absorbance with spectrophotometer.

*2.6 Western immunoblot analysis*

Protein expression was determined by Western immunoblot analysis. About 80-100 μg of proteins were loaded on 8% polyacrylamide gels. The gels lanes were electrophoresed using a Bio-Rad Protein III gel-box onto a nitrocellulose membrane (Bio-Rad: Hercules, CA). Membranes were blocked in 5% nonfat milk with 0.1% Tween-20 in PBS for 6-8 h, and then incubated overnight in room temperature with the appropriate antibody diluted according to the manufacturer's recommendation. Following three washings with PBS with 0.1% Tween-20, horseradish peroxidase (HRP) –conjugated secondary antibodies and an enhanced chemiluminescent (ECL) detection system (Amersham: Picsataway, NJ) were used for visualization. Densitometry was performed using a Nikon camera, a scanner interfaced with a MacIntosh computer, and the NIH Image Analysis 1.61 software program.

*2.7 Antibodies*

Antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) included rabbit polyclonal p-IκB (1:500 dilution), rabbit polyclonal Bax (1:200), and rabbit polyclonal I-κBα (1:200). Antibodies used for Western blot analysis obtained from BD Transduction Laboratories (Lexington, KY) included rabbit polyclonal iNOS (1:7,500), rabbit polyclonal nNOS (1:500), mouse monoclonal NF-κB p65 (1:250), and mouse monoclonal Bcl-2 (1:250). Secondary antibodies were purchased from Santa Cruz Biotechnology.

*2.8 NOS activity assay*

Frozen tissues were homogenized in 6 volumes (w/v) of homogenization buffer (pH 7.4, 10 mM HEPES buffer, 0.1 mM EDTA, 1 mM dithioreitol, 1 mg/ml PMSF, 0.32 mM sucrose, 10 μg/ml aprotinin, 10 μg/ml pepstatin A). The crude homogenates were assayed using a kit produced by Stratagene (La Jolla, CA). The isolated $^{14}C$ citrulline levels were quantified using a liquid scitillation counter. Enzyme activity is expressed in counts per microgram of total protein. Protein concentration was measured by the Bradford technique with BSA as standard (Pierce: Rockford, IL). To differentiate between iNOS activity, which is independent of $Ca^{2+}$ and calmodulin, and constitutive NOS isoform activity, total NOS activity was also measured in the presence of 1.5 mM each EGTA and EDTA, which replaces $CaCl_2$ and calmodulin in the reaction buffer, and in the presence of 1 mM L-NAME (NOS inhibitor). $Ca^{2+}$/calmodulin-dependent NOS activity was calculated as the difference between activities measured in the presence of $CaCl_2$ and that measured in the EDTA/EGTA buffer. $Ca^{2+}$/calmodulin-independent NOS activity was calculated as the difference between samples assayed in the presence of EGTA/EDTA and in the presence of L-NAME.

*2.9 NF-κB DNA binding activity*

We used a sensitive ELISA technique that is specific for activated NF-κB (95, 136). This ELISA kits (Active Motif: Carlsbad, CA) contain a 96-well plate on which has been immobilized oligonucleotide containing the NF-κB consensus site (5'-GGGACTTTCC-3'). The active form of NF-κB contained in sample homogenates specifically binds to this oligonucleotide. The primary antibodies used to detect NF-κB recognize an epitope on p65 that is accessible only when NF-κB is activated and bound to its target DNA. An HRP-conjugated secondary antibody provides a sensitive colorimetric readout that is easily quantified by spectrophotometry. Major procedures involve following steps: 1) fixation of a biotinylated double-strand probe containing the consensus NF-κB binding sequence on streptavadin-coated microplate wells; 2) addition of muscle homogenate samples (20 μg) and positive control (i.e., Jurkat nuclear extract), and incubation at room temperature with mild agitation for 1 h; 3) incubation with rabbit anti-NF-κB antibodies for 1 h without agitation; 4) incubation with horseradish peroxidase-conjugated anti-rabbit antibodies for 1h; and 5) colorimetric development and reading at 450 nm with a reference wavelength of 655 nm.

*2.10 Statistical Analysis*

Two-way analysis of variance was used with Fisher-LSD post hoc to determine if significant effects of aging and exercise training exist among the dependent variables. Significance was set at the 0.05 level.

## 3. Results

### *3.1 Heart / body mass ratio*

In order to determine the efficacy of the exercise training regimen in old and young Fischer-344 rats, heart weight / body weight ratio was assessed as an indicator of training state. Twelve weeks of endurance training significantly increased heart weight / body weight ratio in both 4 mo old (young) and 25 mo old (old) Fischer-344 rats (Fig. 1). Young exercise trained rats had a heart / body mass ratio that was 20.7% higher than sedentary controls. In addition, the heart / body mass ration was 19.1% greater in old exercise trained rats when compared to sedentary controls (Fig. 1).

### *3.2 iNOS protein expression*

Skeletal muscle (tibialis anterior) iNOS protein expression was 118% higher in old rats than in young rats (Fig. 2). However, 12 wk of endurance training in old rats reversed the elevation of iNOS expression. Indeed, trained old rats exhibited iNOS protein expression that was similar to young adults. There were significant effect for aging ($p=0.0073$) and exercise training ($p=0.0006$), but there was no interaction between aging and exercise training ($p>0.1$). In summary, (i) OC was significantly higher (118%) than YC, (ii) YT was significantly lower (62.5%) than YC, (iii) OT was significantly lower (67.7%) than OC, and (iv) OT was not different from YC or YT (Fig. 2).

### *3.3 iNOS and total NOS activity*

iNOS activity was measured in the white gastrocnemius (WG) and soleus (SOL) muscle to determine if age-induced upregulation of iNOS protein levels were manifested in an increase in flux through iNOS. iNOS activity of WG was significantly higher (119%) in old controls

when compared with young controls (Fig. 3). Furthermore, iNOS activity of WG was significantly reduced (-52.0%) by exercise training. In fact, iNOS activity of WG in old trained rats was not significantly different than in young controls. iNOS activity of SOL was also significantly higher in old controls when compared with young controls, and endurance training significantly attenuated the elevation of iNOS activity in old rats (Fig. 4) but not as much as in WG (Fig. 5). There was no difference in iNOS activity between young controls and young trained rats in SOL. Total NOS activity was measured in the WG and SOL (Fig. 6). In both muscles, total NOS activity was significantly increased in young rats when compared with old rats, but there was no training effect in both age groups. In addition, total NOS activity of WG was significantly higher than that of SOL in all groups (Fig. 6).

*3.4 NF-κB DNA binding activity*

We determined NF-κB DNA binding activity as a potential upregulator of iNOS expression. In both young and old groups, binding activity of NF-κB in WG was significantly higher in trained rats compared with sedentary controls and also higher in young groups compared with old counterparts (Fig. 7) that are opposite findings of what we expected. In SOL, there was significant exercise training effect in young groups (Fig. 8) and the same training effects were seen in red gastrocnemius (RG) showing that binding activity of NF-κB was higher in old trained than sedentary controls (Fig. 9). In addition, NF-κB binding activity was significantly increased in young groups. But there was no training effect in young groups. Overall, in WG and RG, NF-κB DNA binding activity was significantly increased with endurance training and was higher in young animals than old animal in both sedentary and trained rats.

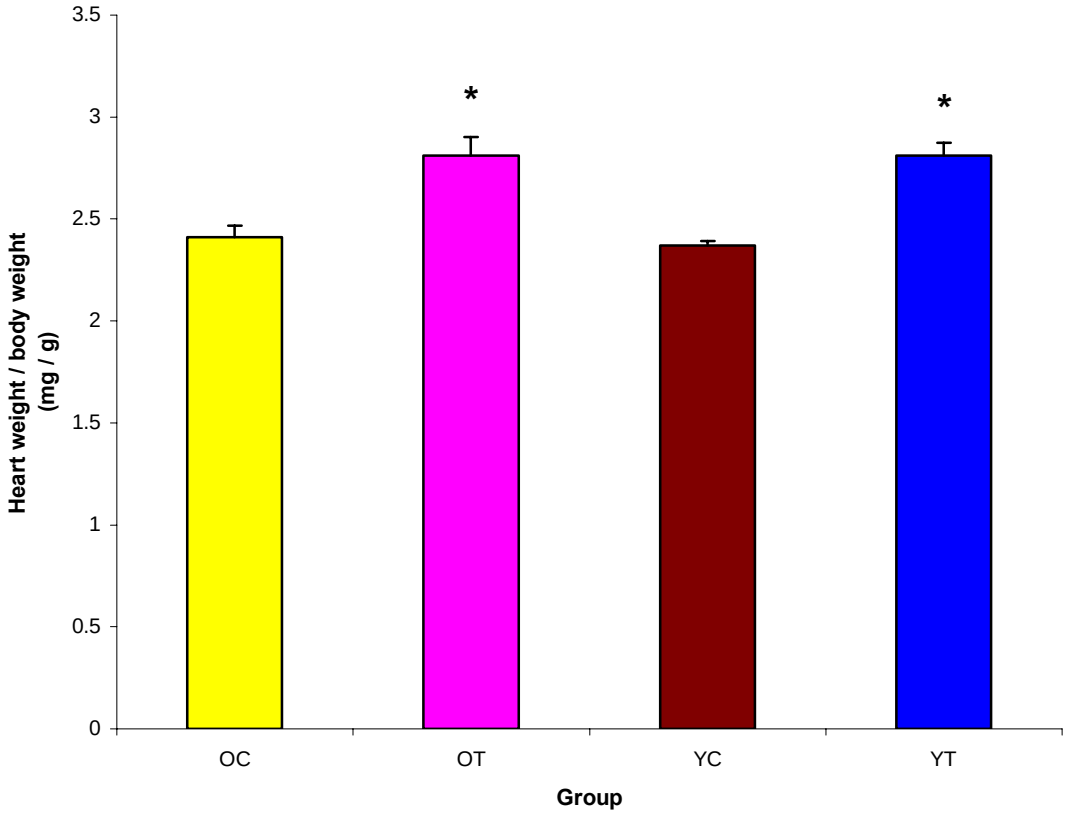


Fig. 1. Heart weight / body weight ratio is shown under four groups. 1) old control (OC), 2) old trained (OT), 3) young control (YC), and 4) young trained (YT). Values are means ± SE. *Significantly different from control within same age group ($p<0.05$).

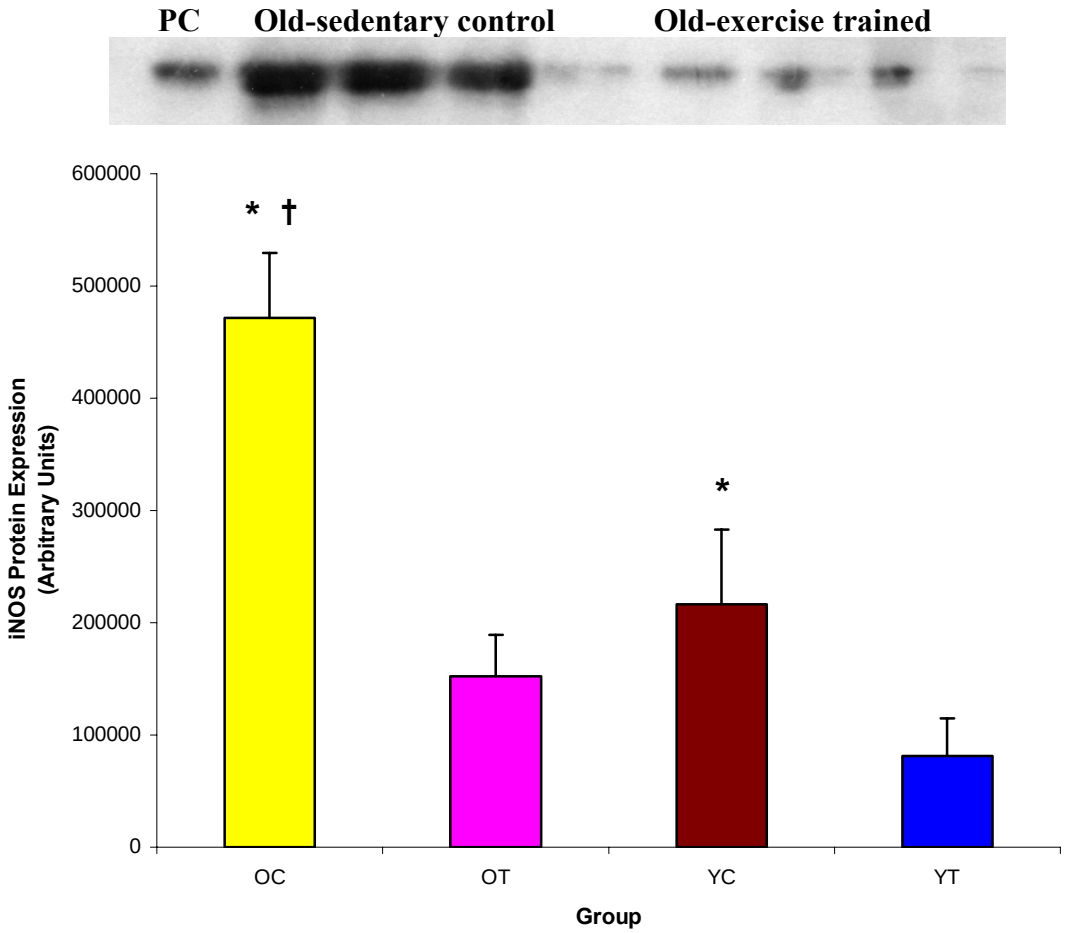


Fig. 2. iNOS protein expression in skeletal muscle (tibialis anterior). Values are means ± SE. PC indicates positive control.*Significantly different from trained within same age group ($p<0.05$). †Indicates that iNOS levels were significantly higher in OC (old control) compared to YC (young control) ($p<0.05$).

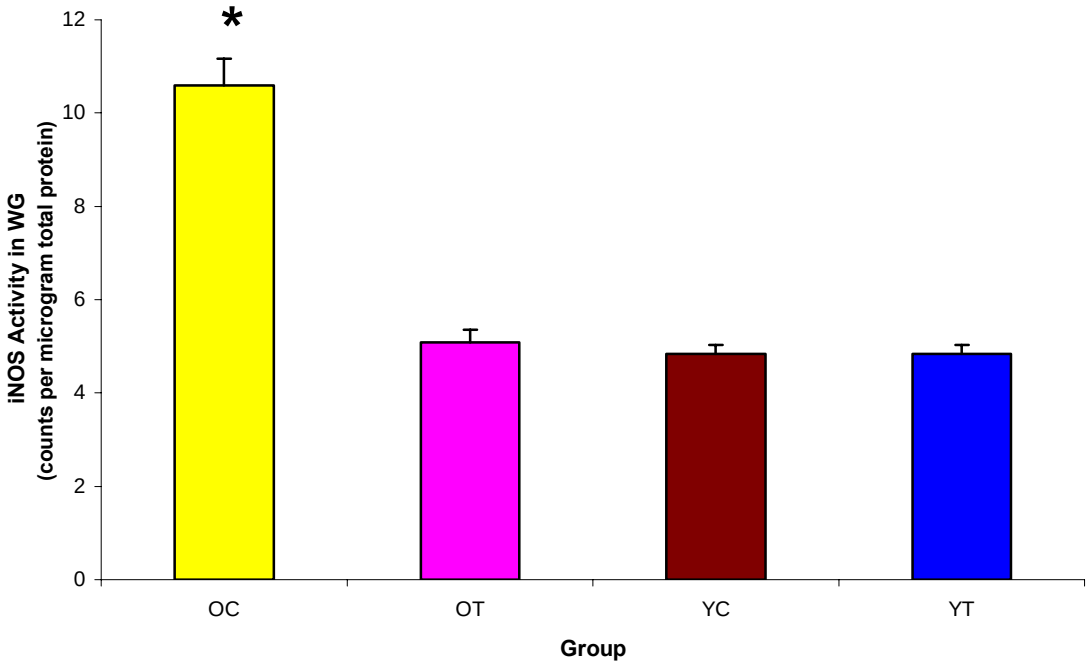


Fig. 3. iNOS activity in white gastrocnemius (WG). Values are mean ± SE expressed as counts/mg total protein. *Significantly different from OT ($p<0.05$). No significant difference between YC and YT ($p<0.05$).

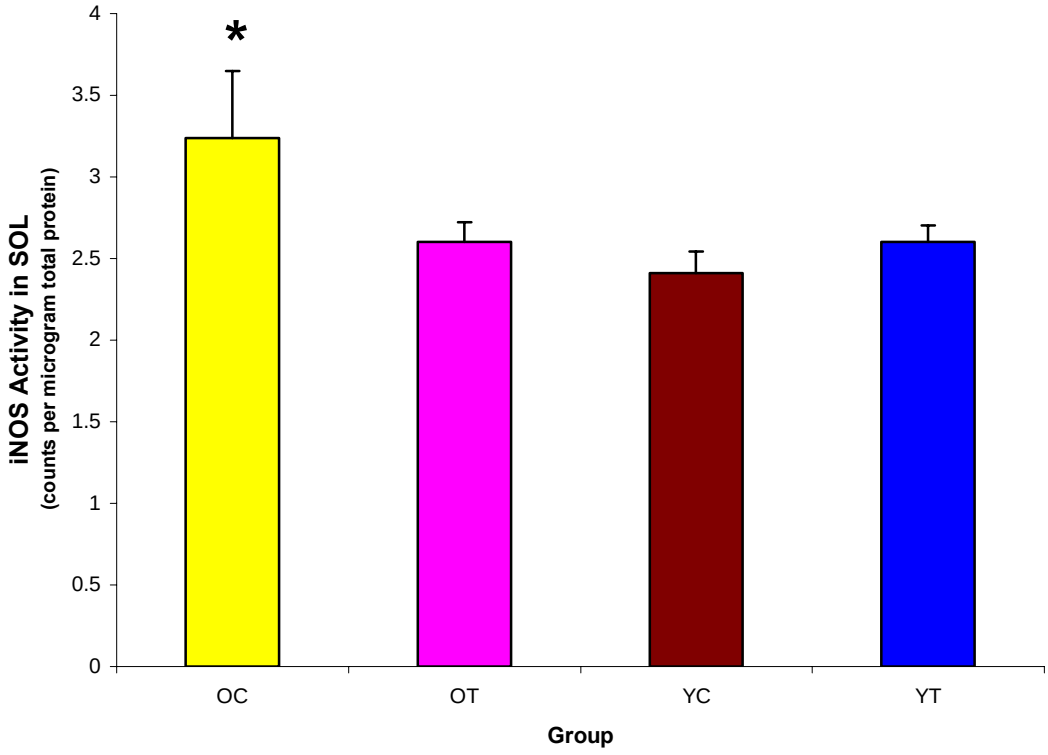


Fig. 4. iNOS activity in soleus (SOL). Values are mean ± SE expressed as counts/mg total protein. *Significantly different from OT ($p<0.05$). No significant difference between YC and YT ($p<0.05$).

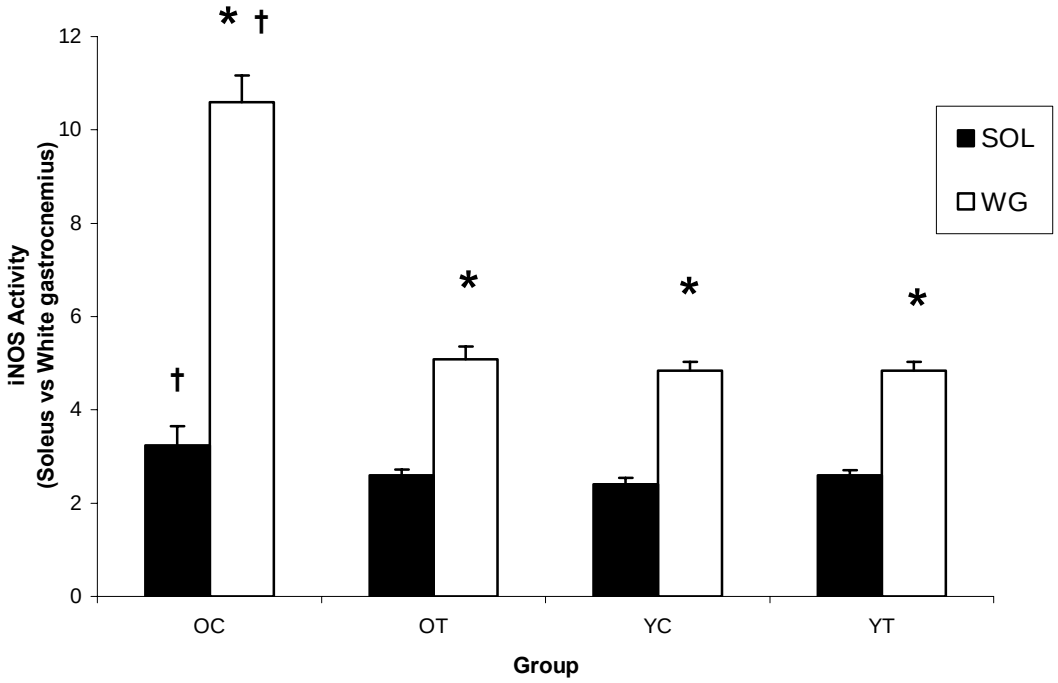


Fig. 5. iNOS activity in soleus (SOL) and white gastrocnemius (WG). Values are mean ± SE expressed as counts/mg total protein. *Indicates that iNOS activity in white gastrocnemius (open bar) is significantly higher than soleus (black bar) ($p<0.05$). †Indicates significantly different from other groups within same muscle ($p<0.05$).

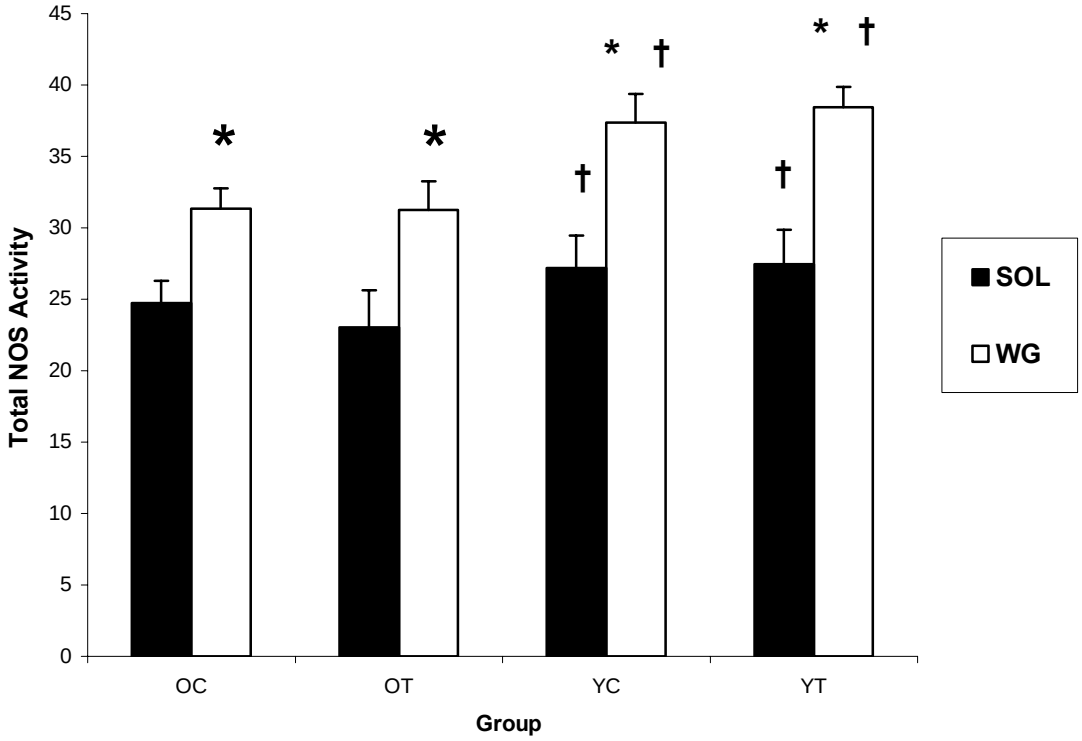


Fig. 6. Total NOS Activity in soleus (SOL) and white gastrocnemius (WG). Values are mean ± SE expressed as counts/mg total protein. *Indicates total NOS activity in white gastrocnemius is significantly different from soleus ($p<0.05$). †Indicates significantly different from OC and OT in both SOL and WG ($p<0.05$).

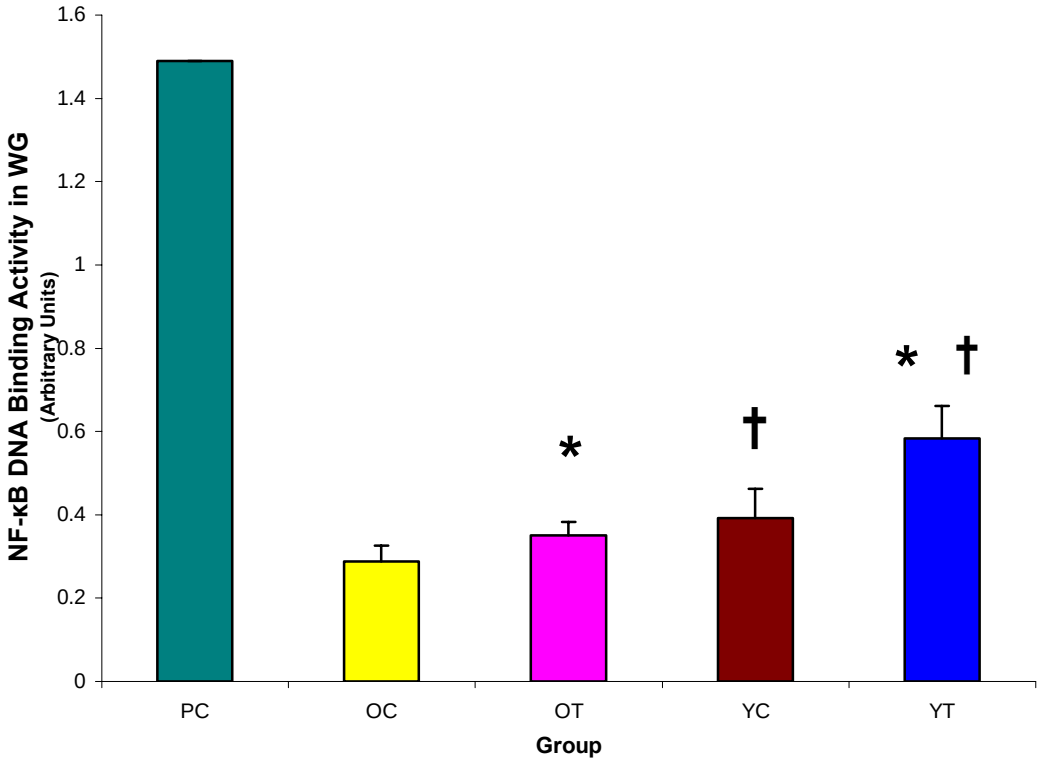


Fig. 7. NF-κB DNA binding activity in white gastrocnemius (WG). Values are mean ± SE. PC indicates positive control. *Significantly higher than sedentary control in each age group ($P<0.05$). †Indicates that activity of YC and YT is significantly higher than OC and OT, respectively ($p<0.05$).

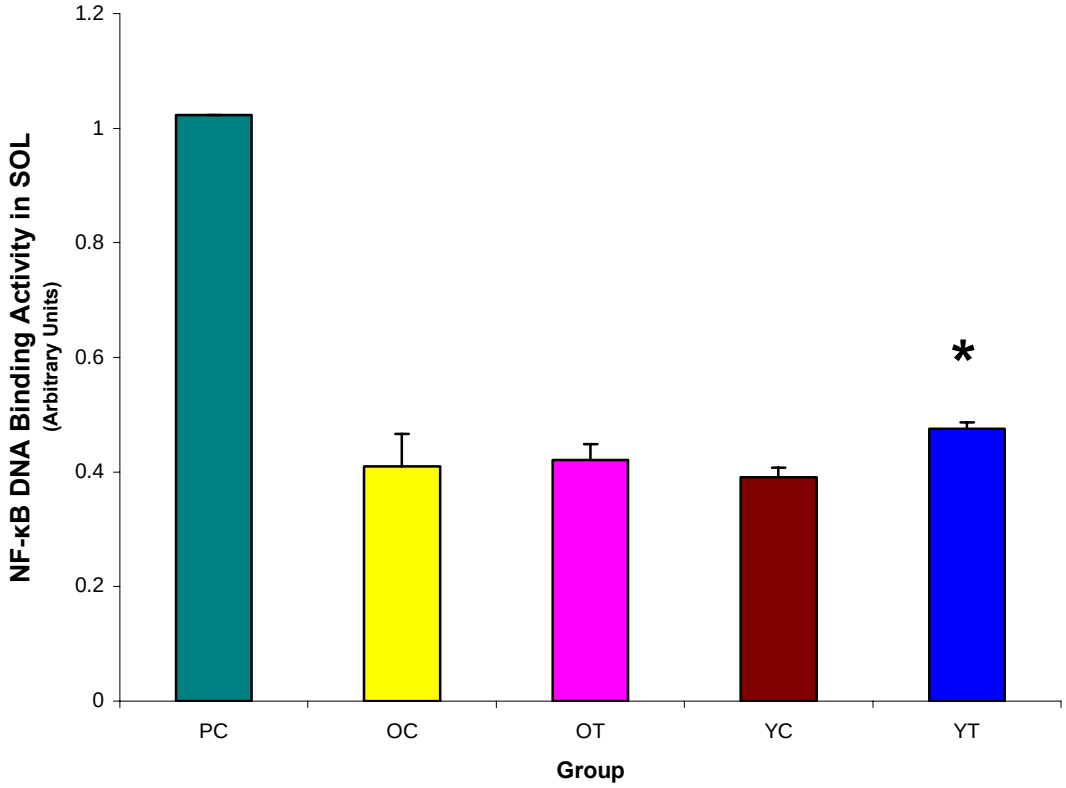


Fig. 8. NF-κB DNA binding activity in soleus (SOL). Values are mean ± SE. PC indicates positive control. *Significantly different from YC ($p<0.05$). No difference between OC and OT ($p<0.05$).

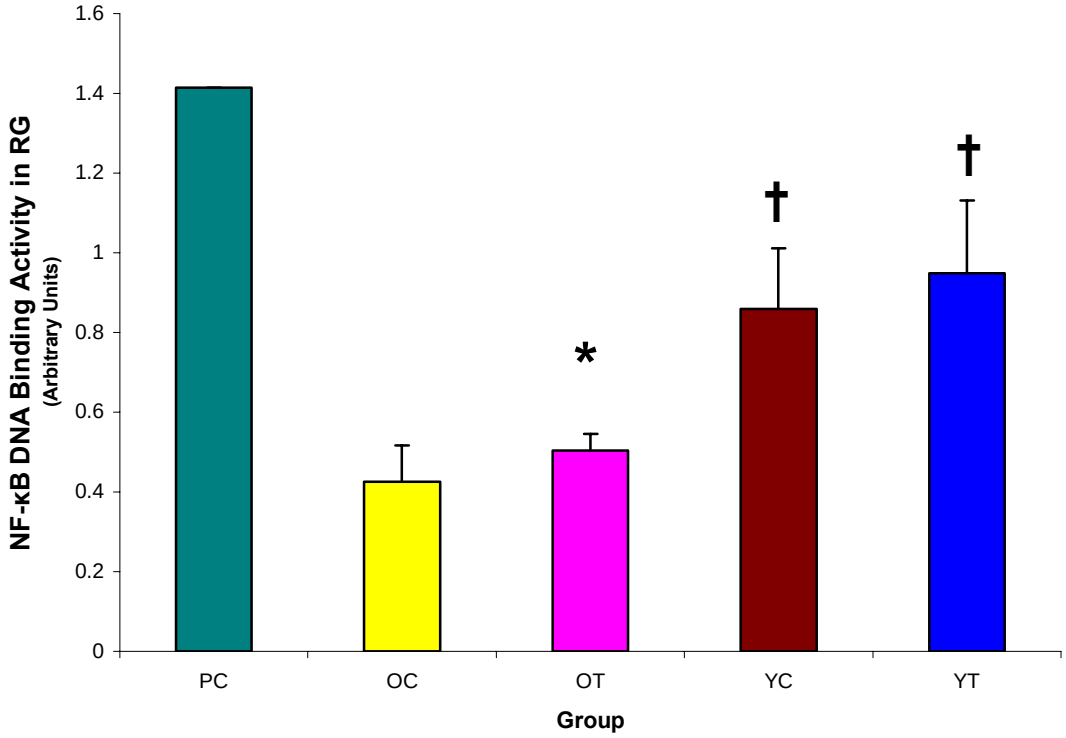


Fig. 9. NF-κB DNA binding activity in red gastrocnemius (RG). Values are mean ± SE. PC indicates positive control. *Significantly different from old sedentary control. No difference between YC and YT ($p<0.05$). †Indicates that activity of YC and YT is significantly greater than OC and OT, respectively ($p<0.05$).

*3.5 I-κBα protein expression*

We hypothesized that aging would decrease I-κBα protein expression in WG and further postulated exercise training would increase cytoplasmic I-κBα providing a mechanism to decrease NF-κB activation. Results revealed a significant decrease (-30%) of I-κBα protein expression with age (Fig. 10). In contrast, exercise training significantly elevated protein levels of I-κBα. Specifically, exercise training increased I-κBα expression by 53.9% in the young rats and 80.2% in the old rats.

*3.6 Phosphorylation of I-κBα*

As phosphorylation of I-κBα is essential for release of active NF-κB, phosphorylation of I-κBα was measured with using phopho-I-κBα antibody in WG. Phosphorylation of I-κBα was significantly higher in young controls when we compared with old sedentary controls (Fig. 11). Exercise training significantly increased (-56.5%) phosphorylation of I-κBα in WG of young rats. In fact, there was no difference between old sedentary controls and old trained rats. The levels of phosphorylation of I-κBα of young trained were significantly higher than that of old trained.

*3.7 p65 protein expression*

If aging decreases NF-κB activity as shown in figures (Fig. 7 and Fig. 9), then it is important to determine whether the regulatory mechanism is through altered NF-κB protein levels or post-translational modifications, so we measured protein expression of p65, part of the dimer that comprises NF-κB in WG, SOL, and RG. p65 levels were significantly increased (45.8%) with aging in WG (Fig. 12). In contrast, exercise training decreased p65 levels in both old (-48.4%) and young adult (-63.4%) Fischer-344 rats. We found same trend of p65 protein

expression in both SOL (Fig. 13) and RG (Fig. 14). p65 levels were significantly increased with aging and endurance training significantly attenuate the elevation of p65 levels in both old and young adult rats of SOL and RG.

*3.8 Anti-apoptotic Bcl-2 protein expression*

Based upon our data from NF-κB DNA binding activity, we postulated that NF-κB exerts anti-apoptotic effect with training and hypothesized that age would reduce anti-apoptotic protein Bcl-2 expression and 12 wk of exercise training would significantly increase Bcl-2 expression in WG. We found that Bcl-2 levels were 20% lower in WG from the old rats compared to the young rats. In contrast, exercise training resulted in a dramatic upregulation (166%) of Bcl-2 in the old trained group when compared to old sedentary controls (Fig. 15). In fact, there was no significant difference between young sedentary controls and young trained rats.

*3.9 Pro-apoptotic Bax protein expression*

In contrast to Bcl-2 protein, we postulated that pro-apoptotic protein Bax expression would increase with aging and exercise training would reduce Bax protein expression thus protecting against pro-apoptotic signaling in skeletal muscle. We found that Bax levels in the WG of old rats were significantly higher (+59.8%) compared to the young group (Fig. 16). In addition, exercise training resulted in a dramatic decrease (-91.5%) of Bax levels in the old trained group when compared to old sedentary controls. Furthermore, exercise training also reduced Bax protein expression in the young group (-21.8%) as compared to young sedentary controls. There was a 70% decrease in Bcl-2/Bax ratio with aging. In contrast, we found a three-fold increase in Bcl-2/Bax ratio by exercise training in the WG from old rats.

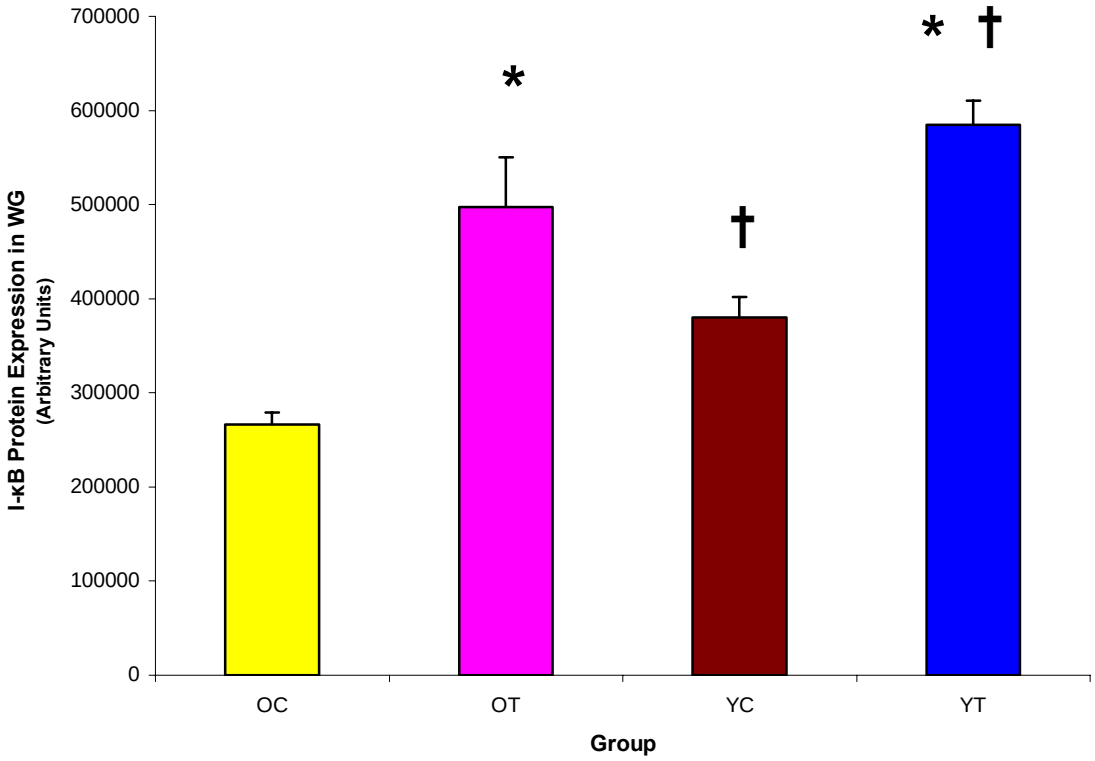


Fig. 10. I-κB protein expression in white gastrocnemius (WG). Values are means ± SE. (*) indicates significantly different than sedentary controls ($p<0.05$). †Indicates I-κB levels of YC and YT are significantly greater than OC and OT, respectively ($p<0.05$).

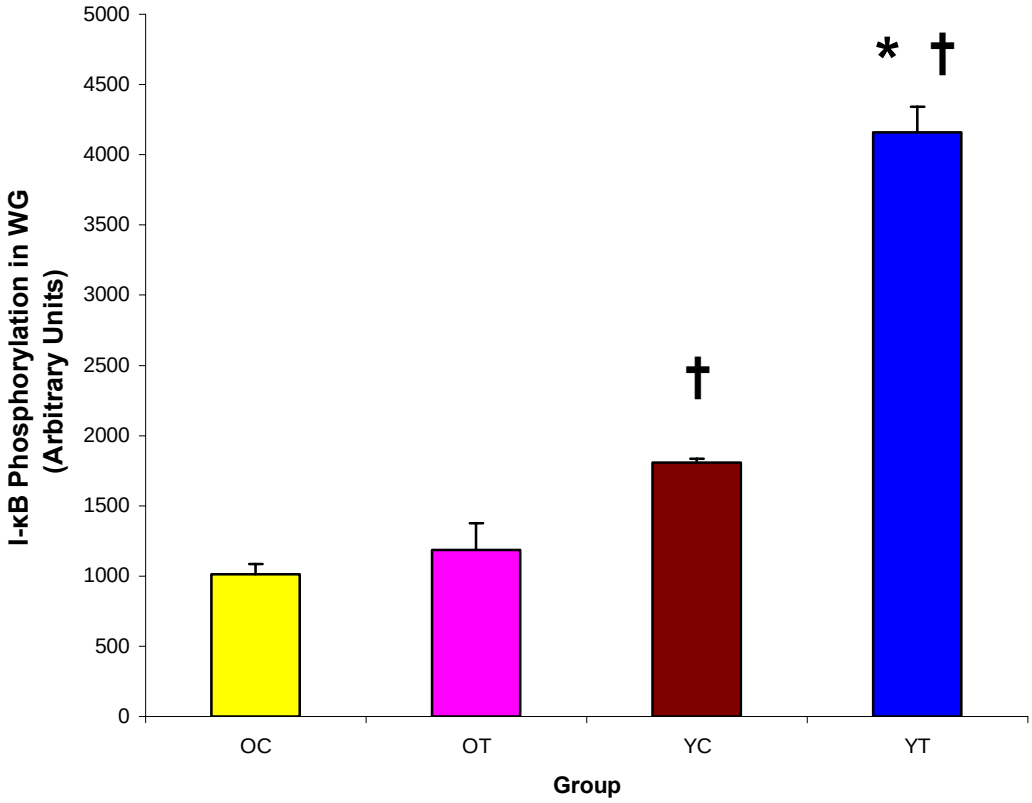


Fig. 11. I-κB phosphorylation in white gastrocnemius (WG). I-κB phosphorylation is measured using I-κB phosphorylation-specific antibody by western blot analysis. Values are mean ± SE. *Significantly higher than controls in young rats ($p<0.05$). †Indicates that I-κB phosphorylation of YC and YT is significantly higher than OC and OT, respectively ($p<0.05$).

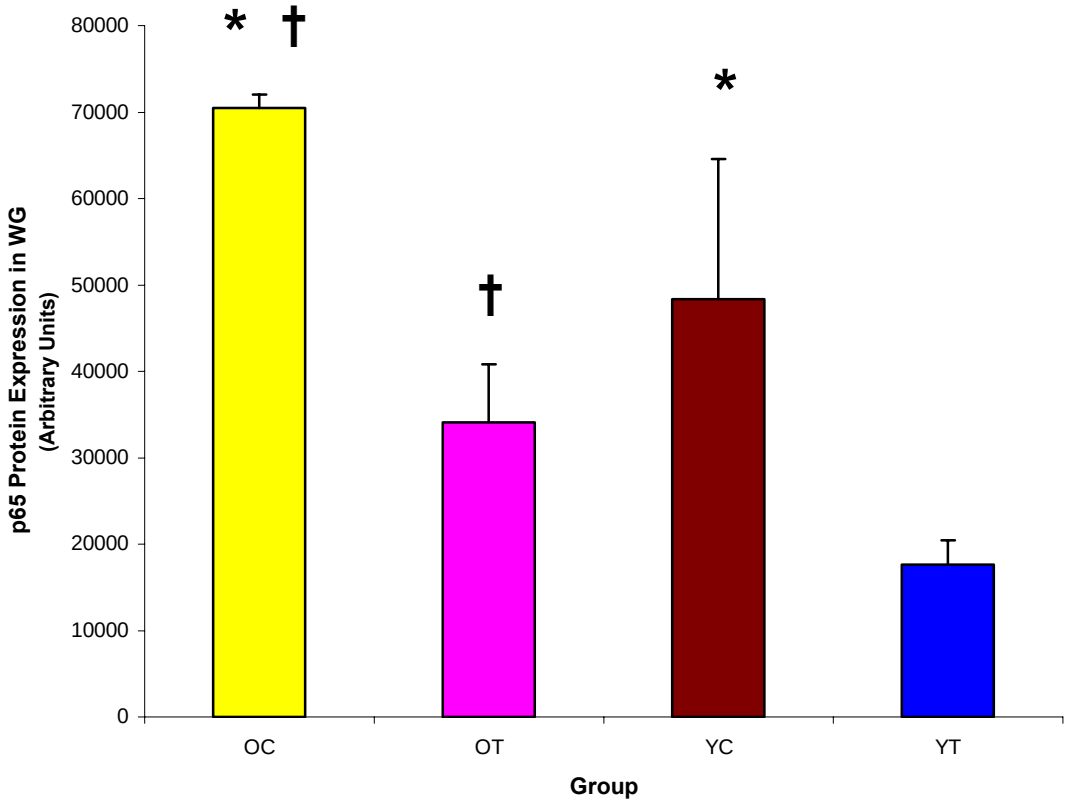


Fig. 12. p65 (NF-κB subunit) protein expression in white gastrocnemius (WG). Values are mean ± SE. *Significantly higher than trained groups ($p<0.05$). †Indicates p65 levels of OC and OT are significantly higher than YC and YT, respectively ($p<0.05$).

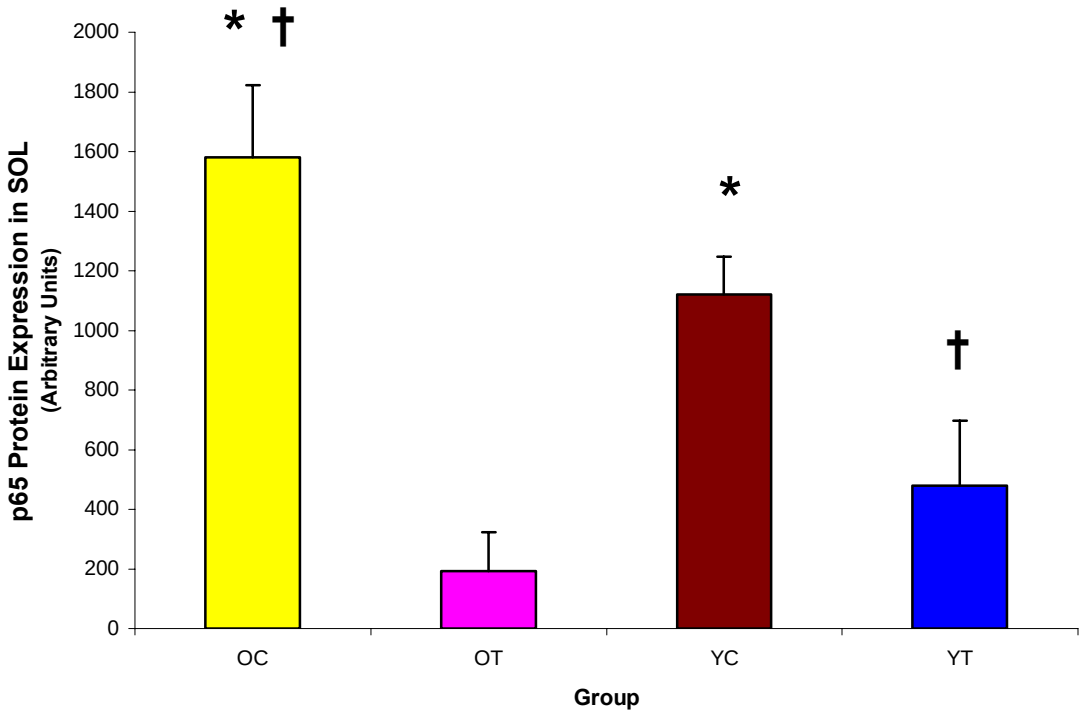


Fig. 13. p65 protein expression in soleus (SOL). Values are mean ± SE. *Significantly different from trained groups ($p<0.05$). †Indicates p65 levels of OC and YT are significantly different from OT and YC, respectively ($p<0.05$).

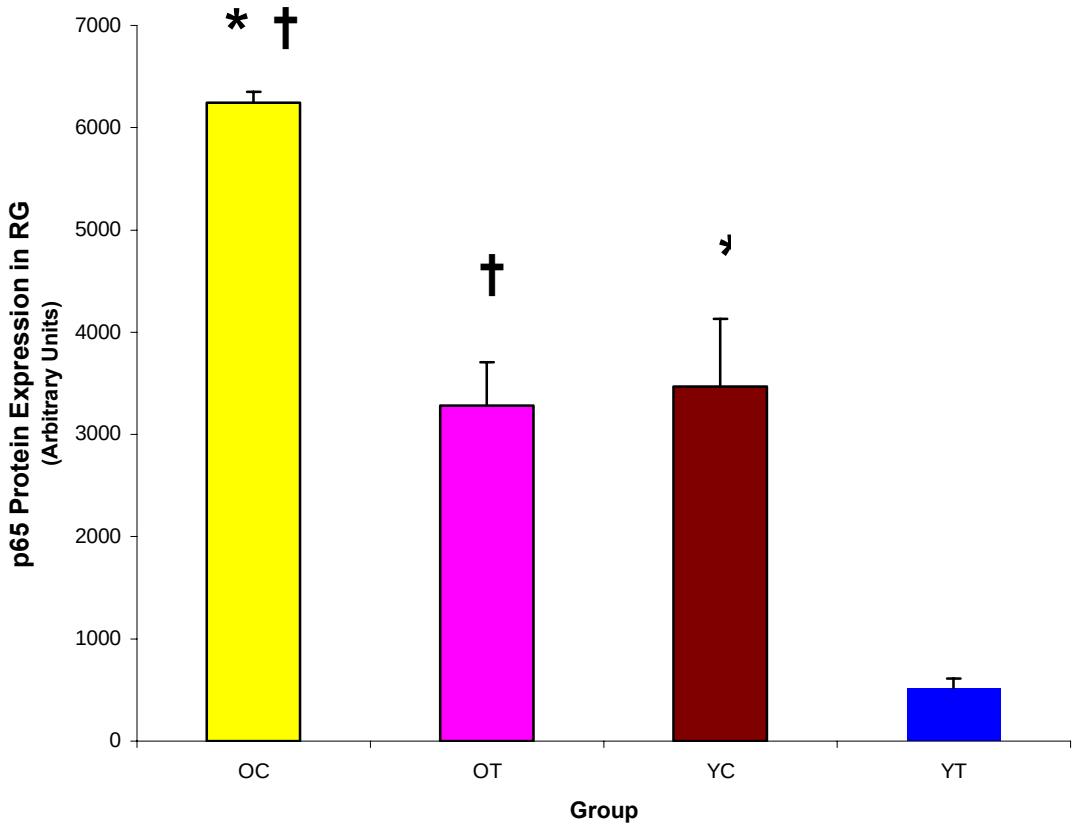


Fig. 14. p65 protein expression in red gastrocnemius (RG). Values are mean ± SE. *Significantly different than trained groups ($p<0.05$). †Indicates that p65 expression of OC and OT are significantly higher than YC and YT, respectively ($p<0.05$).

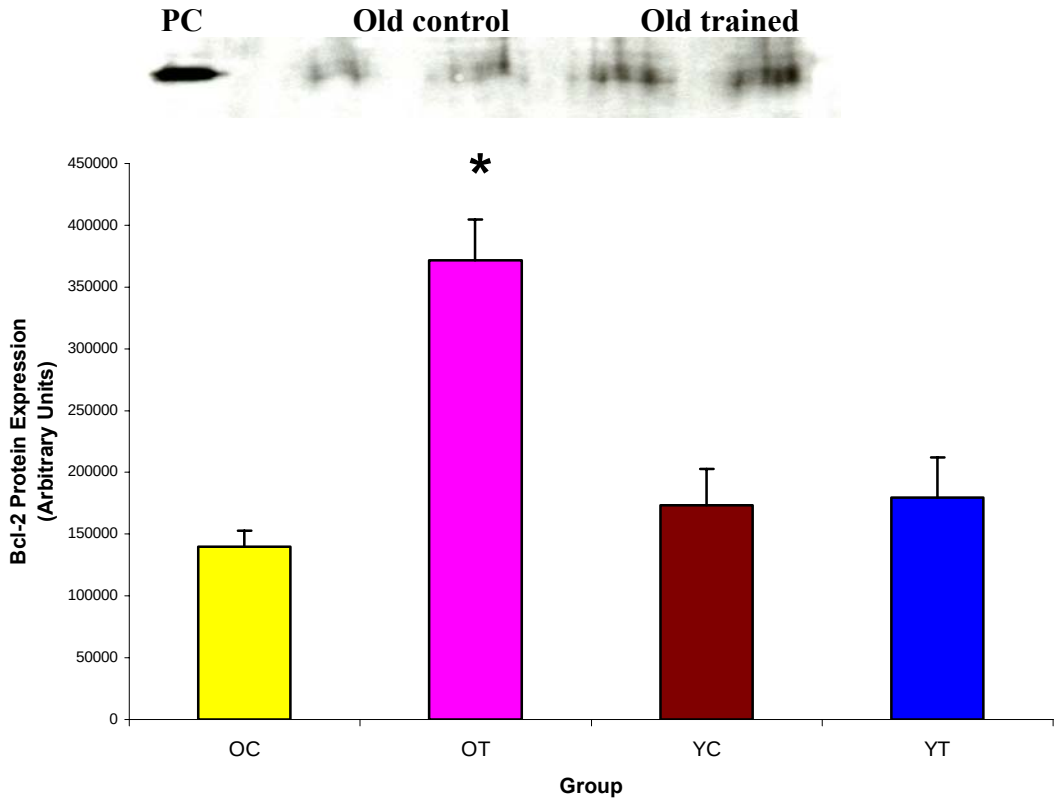


Fig. 15. Anti-apoptotic Bcl-2 protein expression in white gastrocnemius (WG). Values are mean ± SE. PC indicates positive control. *Significantly different from sedentary controls in old rats. No difference between YC and YT ($p<0.05$).

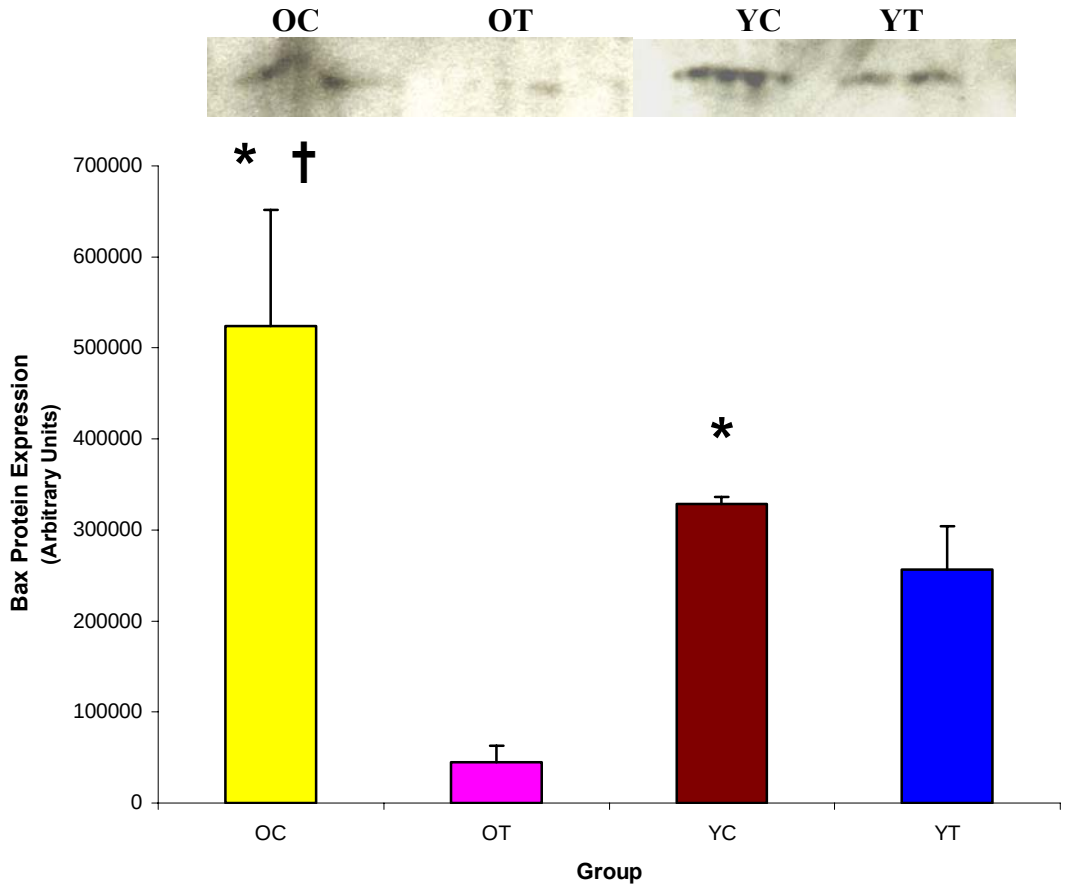


Fig. 16. Pro-apoptotic Bax protein expression in white gastrocnemius. Values are mean ± SE. *Significantly different from trained groups ($p<0.05$). †Indicates OC is significantly different from OT ($p<0.05$).

## 4. Discussion

The novel findings of our study include the following: (i) both iNOS protein expression and activity were higher in old sedentary rats skeletal muscle than in young rats. However, 12 wk of endurance training in old rats reversed the elevation of iNOS levels. (ii) NF-κB DNA binding activity was lower in aged skeletal muscle and exercise training increased NF-κB activity both young and old animals. To the best of our knowledge, this study is the first to demonstrate that endurance exercise training can be a physiological activator of NF-κB DNA binding activity while very little data are reported on the effects of acute exercise. In addition, this study is the first to report iNOS and NF-κB activity based on muscle fiber types including soleus (Type I), white gastrocnemius (Type II), and red gastrocnemius (Mixed). The physiological significance of our findings is discussed below.

### *4.1 Effects of aging and exercise training on iNOS and NF-κB*

#### *1) Effect of aging*

Our present data demonstrate increased levels of iNOS in aged skeletal muscle and support for the global hypothesis that aging increased the pro-inflammatory state, and thus the risk for weakness and muscle atrophy. Evidences shows that pro-oxidant inflammatory pathways become elevated in many tissues as aging progresses (35, 36, 118, 172) and the ability of inflammatory stimuli to induce iNOS, thereby generating large amount of NO, demonstrated iNOS as a major contributor to the chronic inflammatory conditions of aging (36). Several lines of evidence support the idea that iNOS expression is increased with age, including lupus-nephritis-prone mice (87), aged kidney (35), vascular vessel wall (33), and urinary levels of aged animals (132). McCann et al. (117) emphasized the importance of recurrent infections in producing aging changes and iNOS/nNOS ratio was considered as an indicator for nitric oxide

with aging. We measured nNOS protein levels (Fig. 17) and calculated iNOS / nNOS ratio (Fig. 18) showing that iNOS / nNOS is significantly higher in old rats compared to young rats. This data is consistent with the idea that a rise in iNOS with a reduction in nNOS would indicate a shift from a contractile role to an inflammatory role for nitric oxide with aging (117).

Many investigations have explored whether aging affects the regulation of NF-κB because this transcription factor plays a pivotal role in the expression of many genes including iNOS that are central to the inflammatory response (9, 13, 115). Our present results (Fig.7 and Fig. 9) show decreased NF-κB activity in aged skeletal muscle. On the contrary, most current findings show that NF-κB activity is increased with aging, as reported in kidney (86), liver (101), heart (67), and brain (91) tissues. Until now, there was only one report indicating decreased NF-κB activity with aged skeletal muscles, which included soleus, gastrocnemius and superficial vastus lateralis in rats (71). However, our results are different from the previous report as followings. First, we used a highly sensitive ELISA technique to measure NF-κB activity, whereas electrophoretic mobility shift assays (EMSA) was performed (71). Secondly, our data show (Fig. 8) no aging effect in soleus, whereas the Hollander et al. data (71) indicated the most significant decrease in soleus. Our data show differential expression of NF-κB activity in skeletal muscle compared to other post-mitotic tissue such as brain (91) and heart (67). It is likely that alterations in NF-κB activity will be highly cell type and stimulus dependent (58) and more investigations are needed to determine how the aging process regulates NF-κB activation in skeletal muscle.

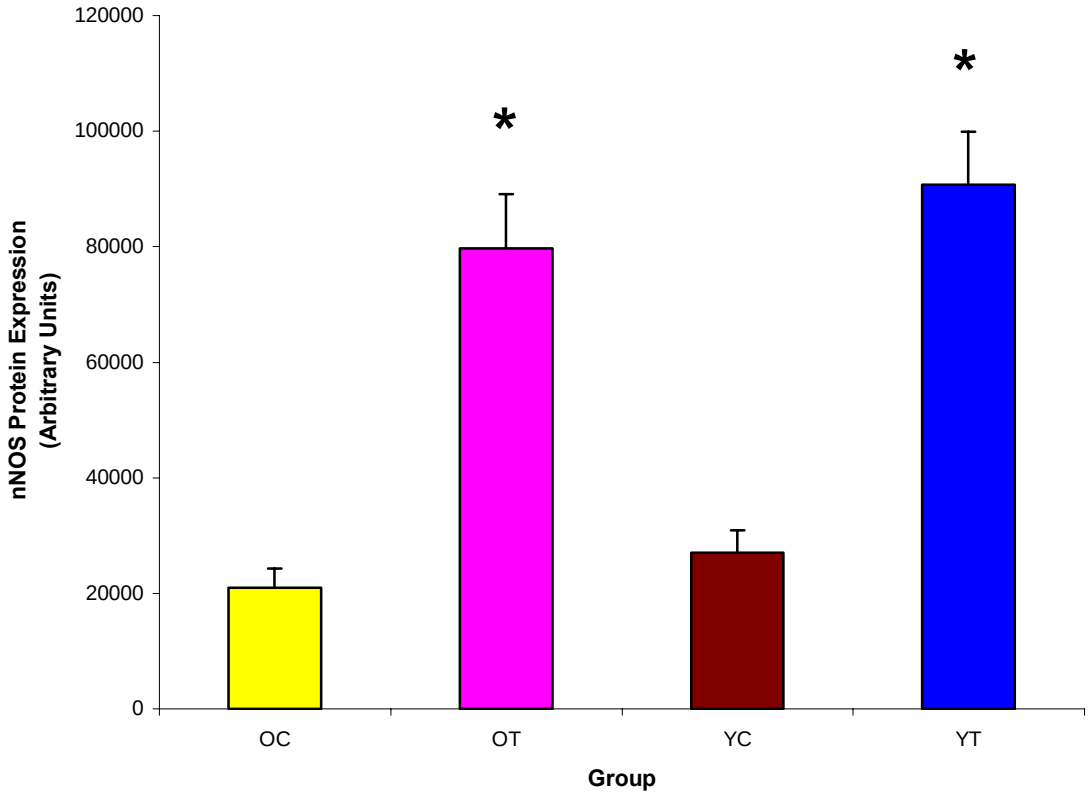


Fig. 17. nNOS protein expression in skeletal muscle (tibialis anterior). Values are mean ± SE. *Significantly different from sedentary controls ($p<0.05$).

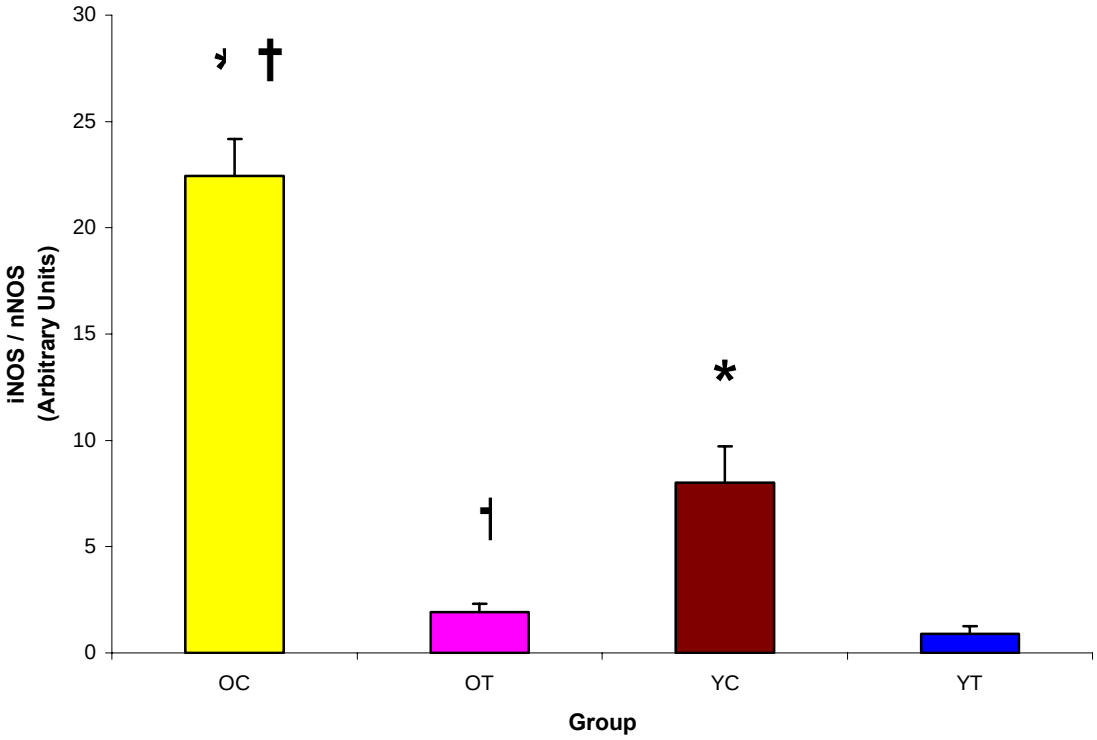


Fig. 18. iNOS / nNOS ratio in rat skeletal muscle. Values are mean ± SE. *Significantly different from trained rats ($p<0.05$). †Indicates ratio of OC and OT is significantly higher than YC and YT, respectively ($p<0.05$).

*2) Effect of exercise training*

We found that 12 wk of endurance training attenuated the elevation of iNOS level in aged skeletal muscle. These results are consistent with the notion that regular physical activity reduces susceptibility of skeletal muscle to inflammation and confirmed the anti-inflammatory effects of chronic exercise. Chronic physical exercise has been shown to play a protective role against inflammation both animals and human (144). For example, LPS-stimulated production of IL-1β and TNF-α was significantly reduced by 5 km running in well-trained athletes (46), and TNF-α release induced by bacterial LPS was also attenuated with exercise training in rats (11). In a recent study Hambrecht et al. (64) demonstrated that local expression of IL-1β and TNF-α in the quadriceps muscle was decreased after long-term endurance training of patients with chronic heart failure. Exercise training appears to reduce local cytokine expression associated with decreased iNOS expression which may in turn contribute to a disinhibition of aerobic enzymes by reduction of intracellular nitric oxide accumulation and protein nitrosylation (144). On the contrary, acute physical activity seems to mediate pro-inflammatory effects. Vigorous exercise ignificantly increased expressions of iNOS and HO-1 at the transcriptional and translational level in human leukocytes as reported by Niess et al (122, 123). This increased expression of iNOS in leukocytes may contribute to an exercise-induced rise of endogenous nitric oxide production and reflects a systemic inflammatory response to exhaustive exercise.

Our present data shows that endurance training increased NF-κB DNA binding activity in skeletal muscle both old and young rats. This is a truly *in vivo* study and there are limited reports regarding effect of exercise on NF-κB activation, because current understanding of NF-κB activation is mostly derived from cell line studies performed *in vitro* condition. Vider et al. (165) provided first evidence to show that acute exercise (1 h) increased NF-κB activation in peripheral blood lymphocytes in young men. Weiss et al. (171) also reported a considerable

activation of NF-κB after 1 h intensive exercise. The mechanism by which acute exhaustive exercise trigger NF-κB activation is possibly through ROS and/or RNS. Decrease of the ratio between intracellular reduced and oxidized glutathione (GSH/GSSG) was accompanied with NF-κB activation indicating the generation of reactive oxygen species during exercise. Additionally, recent report demonstrated that mechanical stretch of skeletal muscle increased NF-κB activation and N-acetylcysteine blocked this activation suggesting free radicals trigger NF-κB activation (99). However, stimuli from mechanical stretch (i.e., *ex vivo*) cannot be considered the same stimuli as exercise training (i.e., *in vivo*) and it is unclear whether mechanical stretch physiologically relevant because the *ex vivo* system is free of neuronal and hormonal effects.

*4.2 NF-κB activation: A possible upstream mechanism of iNOS?*

The present of NF-κB DNA binding activity data refute the original hypothesis of this study that NF-κB would play a role as an upstream mechanism of iNOS expression. As phosphorylation of I-κBα at Ser32 is essential for release of active NF-κB, phosphorylation at this site was measured as an excellent marker of NF-κB activity (53). Consistently, phosphorylation of I-κB also decreased with age (Fig. 11) and confirmed our current results of NF-κB activity. It is also important to note that, in other muscles including SOL and RG, the levels of phosphorylation was not detectable, postulating that I-κBα protein is phosphorylated, and then broken down very rapidly. Thus, capture and detection of this transient form of I-κBα seems very difficult. Underlying mechanisms by which aging and exercise training regulate NF-κB activation would be very complicated and cannot be fully explained just by measuring I-κB phosphorylation and protein levels.

Our results showed that the p65 subunit of NF-κB appears to be not only upregulated by aging but also downregulated by endurance training consistently in WG (Fig. 12), SOL (Fig. 13),

and RG (Fig. 14) and did not correspond with NF-κB activity. These findings suggest that regulatory mechanism is not through changes in expression of protein levels but possibly by post-translational modification. Alternative post-translational NF-κB pathways are the subject of recent findings. Recently, new NF-κB pathways that are not regulated by the I-κB degradation process have been discovered and reported (74, 146). For example, Hutner et al. (76) found that muscle disuse atrophy in young rats is associated with the activation of NF-κB. However, the trigger appears not to be related to or originated from cytokine such as TNF-α-induced NF-κB activation, but rather an alternative NF-κB pathway that does not appear to be associated with inflammation. This novel pathway involves IKKα which is not required for cytokine-induced or inflammation-induced NF-κB activation, but is required for limb morphogenesis, and interestingly p65 activation is not involved (74). Therefore, IKKα is known to be a pivotal component of a second NF-κB activation pathway based on regulated NF-κB (p100) processing rather than I-κB degradation (146).

*4.3 Differential expression based on muscle type (Type I vs. Type IIB)*

Our data indicates that iNOS activity was 119.1% higher in the WG of old control rats compared to young control rats and dramatically reduced by exercise training (-52%). In the same manner, iNOS activity of the SOL was also significantly reduced (-19.6%) by exercise training in old rats but the rate of decrease is not high as we saw in WG (-52%). The WG is composed predominantly of Type IIB (95%) and IID (5%) fibers in adult (5 mo of age) Sprague-Dawley rats (41). This phenomenon can be explained by different rate of muscle atrophy according to fiber types with age. Since atrophy appears to occur in weigh-bearing muscles, and is most remarkable in muscles with a high proportion of type IIB fibers (73), we suggest that

WG, where iNOS activity was the highest, is the most susceptible to inflammation with age among 3 different types of muscles.

Our present data confirmed previous findings by Hollander et al. that NF-κB DNA binding activity of old rat skeletal muscle was 6%, 19%, and 41% lower in superficial vastus lateralis (Type IIB), gastrocnemius (mix), and soleus (Type I), respectively, compared to their young counterparts (71). Specifically, NF-κB DNA binding activity in our data indicate 26.7% decrease in WG and 50.4% decrease in RG of old sedentary rats, and there was no significant reduction in SOL. A possible explanation is that our data just reflect p65 binding activity as a part of subunit of NF-κB making a heterodimer with p50 without considering p50 homodimer's binding activity.

Based on the oxygen cost of running data (105), exercise intensity in our experiment was more than 80% of $\dot{V}O_{2max}$ for old rats and 60% of $\dot{V}O_{2max}$ for young rats. With this intensity old rat needed to recruit type IIB fibers, but it seemed to be not necessary to recruit type IIB fibers due to relative low intensity for young rats according to the size principles. For this reason, we assume that there was a significant difference between old control (OC) and old trained (OT) group but not between young control (YC) and young trained (YT) group in iNOS activity (Fig. 3 & Fig. 4), and relatively smaller difference was seen in SOL than WG (Fig. 5).

*4.4 Exercise training: anti-apoptotic effect on aging skeletal muscle*

Based upon our present findings, given that NF-κB activity decreased with age and increased with exercise training, we wanted to check the possible role of NF-κB in the inhibition of apoptosis in our model. NF-κB is known to influence Bcl-2, and several groups of genes encoding Bcl-2-like proteins have recently identified anti-apoptotic members of the Bcl-2 family (16). In addition, growing experimental evidence suggests that NF-κB activation plays a role as

an anti-apoptotic signal. For example, NF-κB blocks TNF-α-induced apoptosis (19, 181), and inactivation of endogenous NF-κB sensitized cells to stimulus-induced apoptosis (110, 164, 168, 175). Therefore, we tested the hypothesis that exercise training in old rats would protect against pro-apoptotic signaling through increased NF-κB activity in skeletal muscle. Our data show a significant upregulation (166%) of Bcl-2 (Fig. 15) and downregulation (91.5%) of Bax (Fig. 16) by exercise training in old rats. In addition, the Bcl-2/Bax ratio was reduced by 70% with age and increased by three-fold with exercise training indicating that aging muscle is more susceptible to apoptosis than young muscle and endurance training exerts anti-apoptotic action in aging skeletal muscle. Skeletal muscle seems to suffer muscle wasting with advancing age because of both atrophy and loss of muscle fibers (i.e., apoptosis) (131). Recent data confirmed apoptosis in the aged gastrocnemius muscle, where DNA fragmentation was increased by 50% in the old rats compared with the adult animals (45). To our knowledge, ours are the first data to indicate that exercise training has a protective effect against pro-apoptotic signaling in aging skeletal muscle by increasing Bcl-2 levels and by decreasing Bax levels.

The current study provides direct evidence that apoptosis occurs in post-mitotic tissues during the normal physiological aging process, whereas apoptosis has been relatively well documented with pathological conditions such as chronic heart failure (2) and muscular dystrophy (161), in addition to certain non-pathological conditions, for example, hindlimb unloading (6) and acute exercise-induced muscle damage (142). Pro-inflammatory signaling in aging is likely to be complicated and mechanisms by which exercise training exerts anti-apototic influence through NF-κB pathways in aging process should be the subject of future investigation. Measurement of DNA binding activity of other NF-κB subunits, such as p50, will give us better understandings of NF-κB activation with aging and training, and additional investigation of alternative pathway such as IKKα is also strongly desired.

# CHAPTER III

## SUMMARY AND CONCLUSIONS

In summary, the purpose of this study was to (a) determine whether aging affects iNOS expression, and (b) to identify the mechanisms by which exercise training affects aging-induced changes in iNOS signaling in skeletal muscle. The results of present study were the first to demonstrate that both iNOS protein levels and activity were significantly increased in aging skeletal muscle. However, 12 wk of endurance training reversed the elevation of iNOS levels and activity as well. In contrast, age and exercise-induced changes in NF-κB activation, a prime candidate for upstream regulation of iNOS, were not matched with iNOS expression. Specifically, NF-κB activity was decreased with age and increased with exercise training. In contrast, protein expression of the NF-κB subunit p65 was increased in skeletal muscle with aging and decreased by exercise training. These findings suggest that the changes in NF-κB DNA binding activity were post-translational and involved an alternative NF-κB activation signaling pathway. To investigate the possibility that age-related reduction in NF-κB activity promotes apoptotic (i.e., cell death) signaling, we tested the effects of age and exercise training on Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) protein expression. These novel results indicate that exercise training protected against pro-apoptotic signaling in aging skeletal muscle by increasing Bcl-2 levels and decreasing Bax levels.

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Research Assistant, Department of Health and Kinesiology, Texas A&M University, 2000-2003.
International Education Scholarship, Texas A&M University, 2000-2001.
Outstanding Research Presentation Award, Best Overall ERE (Educational Research Exchange) Presentation, College of Education, Texas A&M University. 2002.
1st place Doctoral Presentation, Texas Regional Chapter of ACSM, Georgetown, TX 2002.
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Recognition Award for Meritorious Research by a Young Investigator, the Environmental & Exercise Physiology Section of the American Physiology Society, San Diego, 2003.
Research Manuscript Award, 2nd place, Texas Regional Chapter of ACSM, Houston, TX 2003.
The Korean Honor Scholarship from the Embassy of Korea, Washington D.C. 2003.
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**PUBLICATIONS**

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J.M. Lawler, **W.Song**, S.R. Demaree. Hindlimb unloading increases oxidative stress and disrupts antioxidant capacity in skeletal muscle. *Free Radical Biology and Medicine,* Vol. 35, No. 1, p9-16, 2003.

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